FREE AMINO ACIDS IN CHICK LIVERS AS ALTERED BY VITAMIN B_{12} AND FASTING [1]

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Recent years have witnessed heightened activity in the investigation of free amino acids in biological tissues. This has become of importance in the study of protein nutrition and metabolism. To the authors' knowledge no report has yet appeared regarding the free amino acid content of chick livers. The present investigation was undertaken to explore the possible influence of vitamin B_{12} and fasting on the free amino acids in cockerel livers.

Charkey et al. ('50) have shown that vitamin B_{12} increased chick growth response and at the same time lowered the levels of certain amino acids in the blood. These workers concluded that vitamin B_{12} appears to function in metabolism by enhancing the utilization of circulating free amino acids for building fixed protein tissues. Their observations have been confirmed by Menge and Combs ('50). Sahasrabudhe and Lakshinaroyan Rao ('51) have shown that vitamin B_{12} stimulates protein synthesis in rat livers and presume that this is the result of increased amino acid utilization.

The feasibility of the experiment just cited was made possible by the development of paper chromatography. Awapara

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('49) was among the first to apply this technique to the problem. He described a method of extraction which has been adapted to the present work. Awapara ('48), and Awapara et al. ('50) found free alanine, glycine, aspartic acid, glutamic acid, serine, taurine, glutamine and glutathione in the kidneys, spleens, and livers of Wistar rats. Proline was found in appreciable quantity only in skeletal tissues. Investigators in this field have found a predominance of glycine, alanine, glutamic acid, and aspartic acid in free form in tissues. The "essential" amino acids have seldom been found to occur in significant quantities.

Bull et al. ('49) reported a method of quantitative comparison of amino acids on paper chromatograms by optical scanning. A modification of this method has been used in the present work, as described by Patton and Chism ('51). The method of calculation was that applied to similar work with plant tissues by Payne et al. ('51).

EXPERIMENTAL

Single-comb White Leghorn cockerels from vitamin B_{12}-depleted hens were placed on the basal ration for the first 8 days, then culled and distributed by weight into experimental batteries. At 21 days the first liver samples were taken. The remaining chicks were then fasted 48 hours and liver samples again collected. Final liver samples were taken 4 hours after the fasted chicks had been returned to their respective diets. Each sample contained livers from 5 chicks.

The basal ration contained, in per cent: soybean protein [2] 25, ground yellow corn 70, dicalcium phosphate 3, iodized calcium carbonate 1.61, mineral mixture 0.25, DL-methionine 0.3, choline chloride 0.17, vitamin mixture 0.1 and, in milligrams per kilogram: alpha-tocopherol 2,280, dry vitamin A (3,000 U. per gram) 1,500, dry vitamin D_3 (2,000 U. per gram) 370. The vitamin mixture contained, in grams: riboflavin 6.8, niacinamide 12, calcium pantothenate 7.5, 2-methyl-1,4-naphthoquinone 6.8, thiamine hydrochloride, pyridoxine, *p*-aminobenzoic

[2] Sodium proteinate, Archer-Daniels-Midland Co.

acid and pteroylglutamic acid 3.4 each, mixed with starch 632. The mineral mixture contained, in grams: NaCl 10,000, $MnSO_4 \cdot H_2O$ 338.5, $FeCl_3 \cdot 6H_2O$ 212.5, $CuSO_4 \cdot 5H_2O$ 17.2, and $Co(CH_3COO)_2 \cdot 4H_2O$ 1.9. As supplements to this basal diet, crystalline vitamin B_{12} and animal protein factor (APF)[3] were mixed in the feed at levels to provide 50 μg of vitamin B_{12} activity per kilogram of feed.

Preliminary trials demonstrated a rapid release of free amino acids in chick tissues if enzymes were not promptly inactivated. In order to prevent autolysis, the livers were excised immediately after decapitation and bleeding, and plunged into boiling water. The hot mixture was homogenized in a Waring Blendor, and boiling was continued until foaming ceased. The samples were then stored at — 10°F.

For extraction, samples were thawed, homogenized, and adjusted to 500 ml. Dry matter determinations were made on 50 gm aliquots. Aliquots calculated to contain 1.5 gm dry matter were then centrifuged, and the supernatant liquid decanted with washing and evaporated at 70°C. to 30 ml. To each 30-ml sample was added sufficient ethanol (160 ml) to achieve a final concentration of 80%. The solution was heated almost to boiling, and the precipitated protein was removed by filtration. The filtrate was cooled. After adding 160 ml chloroform and 20 ml water, the mixture was shaken in a separatory funnel and allowed to stand at least 6 hours.

By two-dimensional paper chromatography of such extracts it was established that the upper (aqueous) layer contained in solution practically the entire amounts of those amino acids present in the extract. Proline, which is soluble in the chloroform layer, was not present. The aqueous layer was separated and evaporated at 70°C. to a dry residue. One milliliter of 10% 2-propanol was added to the residue, which was allowed to stand at least 4 hours, warmed slightly to assure complete solution of the amino acids, and centrifuged if necessary to remove insoluble material. Aliquots of 0.1 ml of the clear liquid were

[3] Merck, number 3.

then diluted to 0.2 ml with 10% 2-propanol for paper chromatography.

RESULTS

A ninhydrin-reacting substance appeared on the chromatograms which has been designated as "liver peptide." The chromatographed liver peptide was removed from the paper by elution and hydrolyzed with 6*N* HCl in a sealed tube for 6 hours at 15 pounds. The hydrolysate of this eluate appeared to contain aspartic acid, glutamic acid, glycine, cysteine and phosphorus. The effects of fasting are shown in table 1. Following the feeding of the basal diet, glutamic acid and taurine decreased, alanine increased, and the liver peptide practically disappeared. However, following the feeding of the basal diet plus crystalline vitamin B_{12}, liver levels of glutamic acid and taurine increased instead of decreasing, and alanine increased markedly. At the same time the liver peptide, while dropping appreciably, failed to drop to the vanishing point as it did while the chicks fasted following the feeding of the basal ration. Following supplementation with APF instead of crystalline vitamin B_{12}, similar results were again observed for alanine and liver peptide. In this case, however, glutamic acid decreased instead of increasing, and taurine showed no significant change.

The effect of vitamin B_{12} on recovery from fasting was tested by analyzing samples collected 4 hours after the original diets had been returned to the chicks. The results are shown in table 1. Glutamic acid increased on both the basal and basal-plus-APF diets. However, glutamic acid decreased on the basal-plus-B_{12} diet, following its unusually high level during fasting. Returning to feed caused an increase in alanine on the basal diet, but a marked decrease on the supplemented diets. Similar, but less marked, changes occurred in taurine levels. The liver peptide levels increased in all cases. All differences shown in table 1 were found to be significant at the 0.01 level except in the case of liver taurine with and without the APF-supplemented diet.

TABLE 1

Effect of fasting and returning to feed on non-protein ninhydrin-reacting substances in cockerel livers

SUBSTANCE	OPTICAL DENSITY × 10^3, MEAN OF 10 SAMPLES EACH			COMPARISON OF ON FEED AND OFF FEED		COMPARISON OF OFF FEED AND RETURN TO FEED	
	1. Full feed	2. Off feed	3. Return to feed	Difference between 1 and 2	S. D.[1]	Difference between 2 and 3	S. D.[1]
			B_{12}-Deficient basal				
Alanine	294	328	486	34	3.82	158	3.52
Glutamic acid	915	752	1,030	163	6.06	278	7.12
Liver peptide	827	50	384	777	3.23	334	1.57
Taurine	780	662	877	128	4.41	215	9.04
			Basal diet plus crystalline B_{12}				
Alanine	246	562	50	316	2.96	512	2.30
Glutamic acid	995	1,259	862	264	6.75	397	8.20
Liver peptide	1,577	529	600	1,048	9.21	71	8.43
Taurine	791	871	665	60	4.72	206	2.39
			Basal diet plus APF 3				
Alanine	369	661	281	292	4.86	380	6.10
Glutamic acid	1,176	997	1,045	179	9.42	48	9.56
Liver peptide	1,396	627	1,045	669	5.33	324	7.33
Taurine	731	735	564	4[2]	5.39	171	6.71

[1] S. D. = $\sqrt{\text{Error of variance}}$.

MSD = t value × $\sqrt{\frac{2 \times \text{error of variance}}{\text{number of variants in mean}}}$.

[2] Only difference not significant at 0.01 level according to the method of Villars ('51).

DISCUSSION

The results clearly indicate an important role of vitamin B_{12} in some metabolic process involving the substance designated as liver peptide. On a diet deficient in vitamin B_{12} the level of this substance in the liver was low, and it practically disappeared during fasting. On the other hand, when vitamin B_{12} was present in the ration, liver peptide was maintained at a high level, which dropped but did not disappear during fasting.

The similarity of the liver peptide to glutathione has not been overlooked. Edwards and Westerfield ('52) recently reported that omitting protein from the diet causes a decrease in the glutathione level in rat livers. This might correspond to the observed decrease in liver peptide during fasting. The location of the liver peptide on two-dimensional chromatograms was similar to that of glutathione. However, the liver peptide failed to give a positive nitroprusside test. The appearance of glycine, glutamic acid, and cysteine after hydrolysis also suggests the presence of glutathione. It is not known whether the aspartic acid found upon hydrolysis was present as part of the peptide, or in free form. It is possible that the peptide held back the small amount of aspartic acid present in the liver extracts so it could not rise to the expected position on the chromatograms; such action of peptides has been observed in paper chromatography.

Stekol et al. ('51) found that vitamin B_{12} deficiency impairs cysteine synthesis in rats. This would be expected to result in lower concentration of a compound containing cysteine, as was observed in the case of the liver peptide.

The positive test for phosphorus is unexplained. Rouser and Tishkoff ('51) identified phosphoaminoethanol in rabbit livers by means of paper chromatography. Its two-dimensional location was also in the region occupied by the liver peptide.

The fact that alanine levels generally moved in a direction opposite to those of liver peptide probably has metabolic significance. The observed shifts in glutamic acid and taurine may also be of significance. Glycine and threonine appeared on many chromatograms, but at levels too low to be measured.

No other ninhydrin-reacting substances were distinguishable. The APF was fed at a level that had the same vitamin B_{12} activity as 50 μg of crystalline vitamin B_{12}. However, the APF supplement was an impure substance probably containing many unknown qualities. It is to be expected that it would yield different results than those obtained with the crystalline vitamin.

The results observed are not a peculiarity of the soybean protein diet, since similar results were obtained when the work was repeated using a peanut cake ration.

SUMMARY

Paper chromatograms of protein-free extracts from chick livers contained spots which were identified as alanine, taurine and glutamic acid. Traces of glycine and threonine were also found. An unidentified spot, below glutamic acid, when isolated, hydrolyzed, and rechromatographed, showed the presence of aspartic acid, glutamic acid, glycine, cysteine and phosphorus. The liver level of this substance, apparently a peptide, was markedly increased by the addition of vitamin B_{12} to the diet. Quantitative differences in other amino acid compounds influenced by vitamin B_{12} and fasting were also noted.

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AMINO ACIDS IN THE PRODUCTION OF BLOOD CONSTITUENTS IN RATS

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Previous work in this laboratory (Kornberg et al., '46) indicated that granulocytopenia as well as anemia developed in rats given a diet low in protein. The granulocytopenia was corrected by a combination of protein (casein) and folic acid but not by either alone. It was later shown (Kornberg, '46) that for successful therapy a mixture of the 10 "essential" amino acids could replace the casein; none of the 10 was dispensable except arginine, which appeared to be essential in only about half the animals. Survival of the treated animals was poor, possibly due to the prolonged period of complete protein deprivation used in the development of the blood defect. To obviate this difficulty somewhat, the weanling rats used in the present study were depleted of protein stores by daily bleeding during a short period (5 days) of protein deprivation. The influence of amino acid mixtures in restoring the blood constituents has been studied.

EXPERIMENTAL

Animals and depletion diet

Male rats of the Osborne and Mendel strain approximately 4 weks of age were used. They were fed a protein-free diet (1055, table 1) for a period of 5 days. On each of the first 4 days of this diet, the animals were bled approximately 2% of their body weight.

TABLE 1

Composition of diets

CONSTITUENTS	DIET	
	Protein-free	Amino acid
	%	%
Glucose (anhydrous)	86.76	69.82
Hydrogenated vegetable fat (Crisco)	8.00	8.00
Salts (modified Osborne and Mendel)	4.00	4.00
Ferric citrate [1]	1.16	1.16
$CuSO_4 \cdot 5H_2O$ [1]	0.08	0.08
L-arginine-HCl		1.14
L-histidine-HCl		0.56
L-lysine-HCl·H_2O		1.91
DL-methionine		0.63
DL-phenylalanine		1.40
DL-isoleucine		1.44
DL-leucine		3.24
DL-threonine		1.40
DL-tryptophan		0.81
DL-valine		2.88
Total amino acids		(15.41)
$NaHCO_3$		1.53

VITAMINS [2]	DIET	
	Protein-free	Amino acid
	/100 gm	/100 gm
Thiamine HCl	1 mg	1 mg
Riboflavin	2 mg	2 mg
Pyridoxine HCl	1 mg	1 mg
Ca Pantothenate	4 mg	4 mg
Niacinamide	2 mg	2 mg
Choline Cl	200 mg	200 mg
Biotin	0.001 mg	0.001 mg
Menadione	0.4 mg	0.4 mg
Folic acid		2.5 mg
Vitamin A	[3]	13,000 I.U.
Vitamin D	[3]	1,300 I.U.

[1] Excess iron and copper were supplied because the animals were subjected to repeated hemorrhages.

[2] Once each week all animals were given 3 mg α-tocopherol in 0.03 ml ethyl laurate.

[3] During the period on the protein-free diet, animals were given twice weekly 0.25 ml of corn oil containing 2,000 I.U. of vitamin A and 200 I.U. of vitamin D (Natola).

Bleeding

Blood equal to about 8% of the rat's body weight was removed during the protein-free diet period. In order to avoid any symptoms of shock, the rats were injected intraperitoneally with a volume of 0.85% saline (NaCl) equal to the volume of blood to be removed (Rosenthal and Tabor, '45). The saline was injected just prior to each bleeding.

The bleeding technique used previously (Kornberg et al., '44) was employed with slight modifications. Each animal was placed in a mailing tube of appropriate size with its tail protruding through a hole in the cover. A disk of blotter paper was fitted into the lid to prevent urine from entering the blood-collecting tube. The rat's tail was warmed for three minutes or longer in a constant temperature water bath maintained at 45°C., the end was cut off with a sharp blade and the tail was then immersed in a 1.4% potassium oxalate solution contained in a 10-ml graduated cylinder, which was suspended in the water bath kept at 45°C. When the calculated amount of blood had been withdrawn, the tail was tied near the end to prevent further bleeding. With this procedure, only 4 to 5% of the animals were lost during the depletion period, most of the deaths occurring while the animal was in the container prior to actual bleeding.

Analytical methods

On the day following the 4th bleeding, blood was collected for hematocrit determinations, red, total white and polymorphonuclear cell counts, and serum protein and hemoglobin determinations. The procedures used for all but the last two determinations are those described in a previous publication (Kornberg et al., '44). Hemoglobin was determined by the oxyhemoglobin method of Sanford et al. ('33), and the plasma proteins were determined by the specific gravity method of Lowry and Hunter ('45).

Test diets

The depleted rats were placed on the amino acid test diets. The animals were distributed so as to make each group as uniform as possible. All animals with hematocrits greater than 22% or with a polymorphonucleocyte count greater than 1,500 were discarded.[1]

The test diets differed from the protein-free diet in that a mixture of amino acids plus sufficient sodium bicarbonate to neutralize the hydrochlorides replaced an equal weight of glucose. The amino acid mixture per 100 gm of diet is shown in table 1. All the amino acids were included in the control diet. The other 10 test diets were similar except that one of the amino acids was omitted, and replaced by an equal weight of glucose. Vitamins A and D and folic acid were incorporated into the amino acid diets at the levels shown in table 1.

The rats were maintained on the test diets for 10 days and were weighed periodically. Blood determinations as above were made after 4 and after 10 days on the amino acid diets.

RESULTS

After 4 days on the protein-free diet, during which the rats were bled every day, they were severely anemic, as is shown in table 2.

The addition of the 10 essential amino acids to the protein-free ration produced an improvement in all values measured (table 3). As far as the hematocrit, hemoglobin and red cell counts and serum proteins are concerned, the levels were restored to normal after 10 days on the amino acid-containing ration. Total leukocytes, polymorphonuclear leukocytes and body weight gains were still below normal at that time. All observations made on the 4th day of the amino acid feeding have been omitted from table 3 to save space. The findings show that all values except body weight showed

[1] Less than 1% had hematocrit values greater than 22 and about 8% had polymorphonuclear cell counts above 1,500.

their most rapid rate of restoration during the first 4 days; the rate during the last 6 days was considerably slower. For body weight the reverse was true, with the more rapid rate of gain occurring during the last 6 days. The hematocrit volume increased more rapidly during the first 4 days than did the hemoglobin value. In some cases, the hematocrit remained constant or actually decreased from the 4th to the 10th day, whereas the hemoglobin increased throughout the experiment.

The omission of individual amino acids from the ration had a variable effect on the different values measured. Table

TABLE 2

Observations on groups of rats with and without depletion by multiple hemorrhages

	WITHOUT DEPLETION (Number of rats — 24)		WITH DEPLETION (Number of rats — 110)	
	Average	Range	Average	Range
Hematocrit vol., %	39.7	36–43	14.5	9–22
Hemoglobin, gm/100 ml	12.5	10.6–14.2	4.03	2.8–7.3
Red cell count, millions/mm³	5.7	3.83–7.28	2.19	1.08–3.67
White cell count /mm³	10,050	6,150–15,350	5,070	2,400–9,350
Polymorphonucleocytes /mm³	2,450	800–5,000	760	100–1,500
Serum protein, gm/100 ml	5.38	4.80–6.00	4.11	2.85–5.15
Body weight, gm	46.2	35–61	35.9	25–52

3 indicates that the changes in the hemoglobin and red cell counts paralleled those in the hematocrit. For these three blood measurements, the omission of histidine and valine resulted in a very significant retardation in the rate of regeneration. At the other extreme, the omission of either arginine, methionine or tryptophan had little or no effect.

There appears to have been a reduction in the regeneration of polymorphonuclear leukocytes and of other white blood cells when valine was omitted from the diet. For the regeneration of serum protein, omission of threonine, valine, lysine or tryptophan appears to have had the greatest retarding effects. Average values resulting from the omission

TABLE 3

Observations on groups of rats at beginning and end of amino acid supplementation period. (The significance of the changes when compared with the group receiving all of the amino acids is given by Students' "t" test. The blood values and body weight figures represent averages for 10 animals on each dietary regime)

AMINO ACID OMITTED	HEMATOCRIT			HEMOGLOBIN			RED BLOOD CELLS			POLYMORPHONUCLEAR LEUKOCYTES			OTHER WHITE BLOOD CELLS			SERUM PROTEIN			BODY WEIGHT	
	Days		"t"	Days		"t"	Days		"t"	Days		"t"	Days		"t"	Days		"t"	Days	
	0	10	test	0	10	test	0	10	test	0	10	test	0	10	test	0	10	test	0	10
	Vol. %			*gm %*			*millions/mm³*			*/mm³*			*/mm³*			*gm %*			*gm*	
None	14.2	43.8		3.9	13.8		2.08	6.53		770	1,590		4,540	5,270		4.15	5.20		35.1	44.6
Valine	15.0	29.1	5.04 [1]	4.3	9.2	6.70 [1]	2.36	4.71	4.66 [1]	850	217	5.62 [1]	4,278	2,423	3.71 [3]	4.10	3.70	5.29 [1]	36.4	29.0
Leucine	14.1	34.6	2.17 [2]	3.9	10.6	3.52 [3]	2.12	4.54	3.52 [3]	775	1,095	1.29	4,475	3,810	2.04	4.15	4.75	1.61	35.4	29.6
Tryptophan	14.9	37.7	1.83	4.1	11.6	2.29 [2]	2.23	5.47	1.99	845	1,530	0.75	5,205	4,740	1.54	4.15	3.80	4.66 [1]	38.3	34.5
Histidine	14.8	23.2	5.16 [1]	4.3	6.6	8.44 [1]	2.05	3.65	5.95 [1]	810	1,405	0.50	4,235	4,970	0.03	4.30	4.55	2.86 [2]	37.4	31.6
Isoleucine	14.0	34.9	2.20 [2]	3.8	10.6	3.44 [3]	2.13	5.18	2.40 [2]	800	1,375	0.62	3,850	2,695	3.21 [3]	4.10	4.25	3.42 [3]	35.8	28.3
Threonine	14.4	35.9	2.37 [2]	4.1	12.4	2.47 [2]	2.21	5.95	1.19	675	1,170	0.85	4,195	3,420	2.39 [2]	3.90	3.70	5.30 [1]	37.6	30.3
Methionine	14.2	39.1	1.27	4.1	13.0	1.08	2.19	6.57	0.10	755	710	2.23 [2]	4,570	4,305	1.24	4.10	4.35	2.27 [2]	36.6	28.9
Phenylalanine	14.4	34.4	2.83 [3]	3.9	11.4	2.67 [2]	2.11	5.43	1.79	735	950	1.39	3,900	3,445	1.90	3.95	4.20	3.26 [3]	34.5	27.9
Arginine	14.5	41.1	0.71	4.0	13.6	0.50	2.43	6.20	1.33	770	2,130	0.66	3,705	6,330	1.19	4.20	5.50	0.56	33.7	39.5
Lysine	14.0	31.3	3.15 [3]	4.0	10.1	3.58 [3]	2.07	4.90	2.72 [3]	780	1,735	0.21	3,675	5,570	1.28	4.10	4.05	5.03 [1]	35.8	33.0

[1] Significant at the 0.001 level.
[2] Significant at the 0.05 level.
[3] Significant at the 0.01 level.

of other amino acids together with "t" values are given in table 3.

The absence of any one amino acid other than arginine produced a loss in body weight, while those rats receiving an arginine-free ration gained weight at a subnormal rate. The loss of body weight on the lysine-free ration was relatively small.

Rats on rations deficient in either valine, leucine, tryptophan, histidine, isoleucine or phenylalanine developed tremors after they had been on the diets for 7 to 10 days. Examination of the sciatic nerve and spinal cord from some of the more severe cases has so far failed to reveal any pathology.

It was also noted that rats on the tryptophan-free rations had pale grey stools, whereas the stools of the rats on the other rations appeared normal.

DISCUSSION

The results with the histidine-deficient diet indicate the great importance of this amino acid in hemoglobin and erythrocyte production in the rat. This is not surprising in view of the known high histidine content of hemoglobin. Valine, leucine and lysine also occur in hemoglobin in large amounts (Block and Bolling, '51) and are seen (table 3) to be very important in hemoglobin formation. Isoleucine, however, which appears to be almost as important as leucine or lysine in the restoration of red cell values under our experimental conditions, has been found in hemoglobin (in the species tested) only in very small amounts.

Despite some discrepancies, the correlation between the relative amount of hemoglobin produced on a given deficient diet and that which might have been expected from a consideration of the amino acid composition of hemoglobin, seems surprisingly good. A perfect correlation could hardly have been expected, since most of our animals lost weight during the regeneration period and it has been shown for the dog (Daft et al., '33) that the breakdown products from body protein may be used for hemoglobin synthesis.

It has also been shown that the anemic dog with low plasma protein values shows a preferential production of hemoglobin in the early recovery period (Robscheit-Robbins et al., '43). This appears to have been true for the rats in the present experiments, also. The serum protein values either increased much less than the hemoglobin values, or actually decreased.

It is of interest that valine, in particular, appears to have been of importance for the production of white cells and that threonine, valine, lysine and tryptophan appear to have been of greatest importance in the production of serum proteins.

The results obtained with the arginine-deficient diet are in good agreement with the report of Benditt et al. ('47) that only 9 amino acids are indispensable for the construction of serum protein and erythrocytes in protein-depleted rats.

The tremors exhibited by the rats on the rations deficient in valine, leucine, tryptophan, histidine, isoleucine or phenylalanine differ in some respects from those reported by Rose and Eppstein ('39) for their valine-deficient rats. Rose ('38) reported that nothing similar was observed in rats raised on diets deficient in any other amino acid. In both Rose's work and that reported here, weanling rats were kept on rations deficient in one of the essential amino acids. The rats used in the present work had been on a protein-free ration for 4 days, during which time they had been bled to make them severely anemic. The bleeding may have intensified in some way the development of tremors when the rats were placed on the rations lacking in any one of the 6 amino acids mentioned above.

SUMMARY

1. Rats, shortly after weaning, were depleted of blood proteins and the formed elements of the blood by multiple bleedings while receiving a protein-free diet.

2. The effect of diets deficient in a single "essential" amino acid on recovery of the blood values was studied.

3. Each amino acid deficiency, except that of arginine, had some adverse effect on blood regeneration, but these effects were quite variable.

Histidine, valine and leucine deficiencies had significant effects on the recovery of red cells. Isoleucine and lysine deficiencies also affected the regeneration of hemoglobin, while arginine, methionine and tryptophan deficiencies had the least effects.

Valine deficiency apparently affected the regeneration of polymorphonuclear granulocytes and of other white blood cells, while deficiencies of all of the 10 amino acids tested except arginine, leucine and methionine appeared to affect adversely the regeneration of serum proteins.

4. The animals failed to gain weight on diets deficient in each single amino acid except arginine. The growth of the animals on the arginine-deficient diet was less than on a diet containing all 10 essential amino acids.

5. Tremors developed on rations deficient in valine, leucine, tryptophan, isoleucine or phenylalanine.

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THE EFFECTS OF IODINATED CASEIN, THIOURACIL AND CORTISONE ON THE ASCORBIC ACID REQUIREMENT OF THE MALE GUINEA PIG [1]

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SEVEN FIGURES

(Received for publication November 14, 1951)

It has been established that the requirement of the guinea pig for ascorbic acid is about 0.7 mg per 100 gm body weight, when the height of the odontoblasts and the weight of the adrenal glands are used as criteria (Pfander and Mitchell, '52).

Certain endocrine glands seem to be related to the metabolism of ascorbic acid. Drill ('43) has reviewed the thyroid interrelationships. Recently, Schaffenburg et al. ('50) showed that cortisone reduced some of the symptoms of gross scurvy during a three-week experiment, and Hyman et al. ('50) essentially confirmed those results and extended their observations to include the adrenocorticotropic hormone (ACTH) of the pituitary.

This paper reports the quantitative effects on ascorbic acid requirement observed in groups of guinea pigs treated with iodinated casein, thiouracil, or cortisone. More complete details of the feeding and management of the animals and of the technics employed have been given by Pfander and Mitchell ('52).

[1] The data reported in this paper are taken from a thesis, prepared under the guidance of Dr. H. H. Mitchell, submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

[2] Fulbright Fellow now at the Rowett Research Institute, Aberdeen, Scotland.

EXPERIMENTAL

The effect of hyperthyroidism

Five trios of male guinea pigs weighing between 250 and 330 gm were given daily doses of 20 mg iodinated casein; an amount which was calculated to be equivalent to 0.54 mg of thyroxine. The iodinated casein was mixed with sucrose and the mixture put in number 2 gelatin capsules. The accuracy of filling these capsules was determined to be $\pm$ 0.47 mg of iodinated casein. The initial dose proved to be too high, as the animals stopped eating after about 7 days. The drug was withdrawn for 5 days and then a dose of 6 mg was used for the remainder of the experiment, the total length of which was 7 weeks.

Respiration rates were determined by observation, and heart rates by synchronizing the beat of the heart as heard through a stethoscope with the tapping of a pencil passing over a paper for one minute. An average of 5 series of such counts was recorded as the rate for the day.

The basal diet was a slight modification of the Crampton ('47) scorbutogenic diet. Since hyperthyroidism is known (Drill, '43) to increase the requirement for certain vitamins, the vitamin components of the basal diet were doubled in these experiments. The guinea pigs were fed individually in an air-conditioned room. The food consumption was equalized within trios, the members of which received the following doses of ascorbic acid, in milligrams per 100 gm body weight: 0.70, 0.55 and 0.40, respectively.

The effect of hypothyroidism

Twelve guinea pigs were divided into three groups to receive respectively 20, 40 and 60 mg of thiouracil per kilogram body weight. Within each of these groups, one guinea pig received 0.70 mg of ascorbic acid daily per 100 gm body weight, another received 0.62 mg, another 0.55 and the 4th 0.40 mg. All 12 animals were given the same amount of food daily, the

basal diet being the same as in the preceding tests, including the elevated vitamin content.

Hypothyroidism is thought to decrease the conversion of dietary precursors to vitamin A (Wendt, '35; Wohl and Feldman, '39).

During the 5th week a slight indication of xerophthalmia was observed in one animal, so the vitamin A level for all animals was increased to three times the original level. Ascorbic acid was given in the morning, and thiouracil mixed with sucrose in gelatin capsules was given in the evening.

The effect of cortisone

Schaffenburg et al. ('50) showed that cortisone injected over a 21-day period prevented certain signs of scurvy: in particular, enlarged adrenals and loss of weight.

The effects of cortisone over a 7-week period were studied, using 9 guinea pigs. Each guinea pig received one of each of the cortisone dosages, 0, 2.5, and 5.0 mg per day, and one each of the ascorbic acid dosages, 0.70, 0.55, and 0.40 mg per 100 gm body weight per day. All 9 guinea pigs received the same amount of basal diet.

The cortisone and saline were given by subcutaneous injection. The level of the vitamin component in the basal diet was doubled. At the beginning and end of the experimental period guinea pigs were placed in glass- or plastic-lined cages and 24-hour urine samples were collected into metaphosphoric acid in the dark. The ascorbic acid content was then estimated by the method of Roe and Kuether ('43).

Other procedures were the same as those described previously.

The results were analyzed by the analysis of variance.

RESULTS

The effect of *hyperthyroidism* on the heart rate, adrenal weight and odontoblast height is summarized in table 1. The adrenal size has been increased at the levels of ascorbic acid

studied, and the odontoblast height has been decreased. The control guinea pig receiving 1.4 mg of ascorbic acid per 100 gm body weight and iodinated casein appeared to be normal from the standpoint of ascorbic acid nutriture, but showed all the symptoms of hyperthyroidism shown by the animals on the lower levels; that is, shedding of hair, irritability, and exophthalmos, which persisted even though the vitamin A supplement was doubled. One animal receiving the 0.40-mg level of ascorbic acid died and showed several of the classical symptoms of scurvy, including hemorrhage of the intestines

TABLE 1

Effect of hyperthyroidism

	MG ASCORBIC ACID PER 100 GM BODY WEIGHT				
	1.40 [1]	0.70 [2]	0.55 [2]	0.40 [2]	Natural control [4]
Heart rate per minute	329	339	336	336	266
Adrenal weight, mg per 100 gm body weight	62	102	120	97	58
Odontoblast height, μ	62	43	31	[3]	62

[1] Observation on one guinea pig.

[2] Averages for 5 guinea pigs.

[3] Odontoblasts were disorganized and the height could not be determined.

[4] Received carrots, lettuce, cabbage and grass, in addition to the scorbutogenic diet offered ad libium.

and at the joints of the long bones. The remaining animals on the 0.40-mg level showed the same symptoms to a lesser degree at necropsy. A negative control guinea pig, receiving no ascorbic acid, died in 24 days. Plate 1 shows photomicrographs of the teeth from one trio on this study. Note that the odontoblasts were completely disorganized at the low levels.

Unfortunately the data do not justify a direct quantitative estimate of the ascorbic acid requirement for the increased metabolism due to the treatment with iodinated casein; however, using the available data, one may calculate regression equations of adrenal weight and odontoblast height on dose of ascorbic acid. One may then solve these equations for x

(ascorbic acid dosage) by setting y (odontoblast height or adrenal weight) equal to levels reached by control animals.

For the odontoblasts. $y = 22.4 + 39.4x$; when $y = 62$, then x equals 1.005 mg per 100 gm body weight, where y = odontoblast height in micra and x = intake of ascorbic acid in milligrams per 100 gm body weight.

For the adrenals. $y = 182.7 - 114.7x$; when $y = 58$, then $x = 1.087$, where y = weight of adrenals in milligrams per 100 gm body weight and x = intake of ascorbic acid in milligrams per 100 gm body weight.

From these two calculations, which check remarkably well, one may say that the requirement of ascorbic acid by the hyperthyroid guinea pig is apparently about 1 mg per 100 gm body weight, but less than 1.4 mg. The validity of these calculations is somewhat impaired by the fact that the height of odontoblasts could not be determined at the 0.40 mg level, and the adrenal size was smaller at this level than at the higher levels; therefore, these observations were not used in the calculations. Dann and Cowgill ('35) stated that the fact that they found the requirement to be proportional to body weight would indicate that the rate of metabolism was not a factor in the vitamin C requirement, but that it would be interesting to check the effect of hyperthyroidism.

During the first few days of the experiment on *hypothyroidism,* the thiouracil was given in the food and the animals readily consumed the dose, but as the animals became larger the total daily dose increased and refusals occurred. The drug was then given in capsules. The effect on appetite is shown in table 2. For the first 5 weeks the same response was obtained, but during the last two weeks of the test, the animals on the higher levels of thiouracil limited the intake of all animals on the test.

Table 3 contains the adrenal weights, adrenal ascorbic acid contents and odontoblast heights for the 12 guinea pigs used in the study of the effect of hypothyroidism on ascorbic acid requirements. An analysis of variance of these data indicates that while there may have been a significant effect

of ascorbic acid dosage on adrenal weight ($P = 0.05$), there was no effect of thiouracil treatment ($F = < 1$). The 0.70 mg of ascorbic acid per 100 gm body weight was no more effective in preventing adrenal enlargement than the 0.62-mg level, which would indicate that the requirement was 0.62 mg per 100 gm body weight or less. This amount would seem to be slightly below that required by the untreated animals.

The animals in this experiment behaved in a manner comparable to that of the animals not receiving thiouracil in that the adrenal concentration of ascorbic acid reflected the treatment level ($P = < 0.05$), but was unaffected by thiouracil dosage ($F = < 2$). From daily observation no differences

TABLE 2

The effect of thiouracil level on food refusal

TEST DOSE OF THIOURACIL MG/KG BODY WEIGHT	TOTAL REFUSALS PER GROUP	
	First 5 weeks	Last 2 weeks
20	29	6
40	26	31
60	28	45
Total	83	82
Chi square	< 1	29
Chi square required for P = 0.01 (2 degrees of freedom)		9.21

could be detected in these animals and those not receiving the drug, thiouracil, indicating that the dosage was not producing a marked physiological effect. Unfortunately the thyroids were not examined and the degree of inhibition is not known.

There was no effect of level of thiouracil on odontoblast height ($F = 2.58$, where a value of 5.41 is equivalent to $P = 0.05$), and in contrast to the adrenal data, the 0.62-mg level was not as effective as the 0.70-mg level of ascorbic acid in producing odontoblasts of maximum height in two of three comparisons (see also plate 1). Apparently the levels of thiouracil used had no demonstrable effect on the ascorbic acid requirement.

TABLE 3

The effect of thiouracil on adrenal size, adrenal ascorbic acid and odontoblast height

THIOURACIL DOSAGE IN MG PER KG BODY WEIGHT	MEASUREMENT TAKEN	ASCORBIC ACID DOSAGE IN MG PER 100 GM BODY WEIGHT PER DAY				
		0.70	0.62	0.55	0.40	Averages of rows
20	Adrenal weight, mg per 100 gm body weight	66 [1]	63	68	94	73
	Adrenal ascorbic acid, mg %	55	34	22	8	30
	Odontoblast height, μ	70	60	61	22	53
40	Adrenal weight, mg per 100 gm body weight	54	52	81	84	68
	Adrenal ascorbic acid, mg %	88	30	27	17	40
	Odontoblast height, μ	63	54	35	28	45
60	Adrenal weight, mg per 100 gm body weight	67	65	71	75	70
	Adrenal ascorbic acid, mg %	40	34	19	11	26
	Odontoblast height, μ	67	49	24	10	38
Averages of columns	Adrenal weight, mg per 100 gm body weight	62	60	73	84	
	Adrenal ascorbic acid, mg %	61	33	23	12	
	Odontoblast height, μ	67	54	40	20	

[1] This animal showed severe renal pathology. The values for this animal were obtained from the other data in their respective rows and columns by Fisher's ('44) method of maximum likelihood.

As might be expected, the animals which received *cortisone* had small adrenals. Table 4 shows the values for the ratio of adrenal weight to body weight; the analysis of variance indicates that both increasing doses of cortisone and of ascorbic acid decrease the size of the adrenal gland at a probability level of 5% or less, the cortisone effect being more distinct. Pirani et al. ('51) have reported similar results.

The excretion of a substance measured as ascorbic acid by the Roe and Keuther method is increased by the injection of cortisone (P = approximately 0.01), while at the same time the average adrenal concentration goes down, but the decrease is not significant at the 5% level of probability.

The animals in this experiment seemed little disturbed by the injection process. They ate well most of the time except during the third week, when the temperature of the room rose to 85°F. and the circulation of fresh air stopped for two days.

In addition to the animals in the block design, 4 animals fed ad libitum were carried on the 2.5-mg test dose of cortisone. One received the 0.30 mg per 100 gm level, two the 0.40-mg level, and one the 0.55-mg level of ascorbic acid. The data from these animals are not subject to statistical treatment, but the values are similar to those obtained with animals whose food was controlled.

It was noted that there was a tendency for blood from the animals receiving 5 mg of cortisone to clot in spite of the addition of amounts of oxalate which would prevent coagulation for 24 hours in the other animals. This may be compared with the observation that in human subjects receiving cortisone, coagulation time is shortened and emboli are sometimes formed (Cosgriff et al., '50).

The odontoblasts in the animals on the 5-mg level of cortisone showed two effects. The odontoblasts formed during the initial weeks of the test were large, measuring about 64 μ and 50 μ for the 0.55- and 0.40-mg levels, respectively. This would indicate that the presence of cortisone was lowering the requirement for the first few weeks. The odontoblasts formed in the later weeks of the experiment, however, were

TABLE 4

The effect of cortisone on adrenal size, adrenal ascorbic acid concentration, urinary excretion of ascorbic acid, and odontoblast height

CORTISONE DOSAGE IN MG PER DAY	MEASUREMENT TAKEN	ASCORBIC ACID DOSAGE IN MG PER 100 GM BODY WEIGHT PER DAY			
		0.70	0.55	0.40	Averages of rows
0 (saline injection)	Adrenal weight, mg per 100 gm body weight	59	75	92	75
	Adrenal ascorbic acid, mg %	68	63	36	56
	Urinary ascorbic acid, μg per day [1]	1	1	1	1
	Odontoblast height, μ	63	55	30	49
2.5	Adrenal weight, mg per 100 gm body weight	44	55	60	53
	Adrenal ascorbic acid, mg %	45	35	37	39
	Urinary ascorbic acid, μg per day [1]	9	8	10	9
	Odontoblast height, μ	63	54	46	54
5.0	Adrenal weight, mg per 100 gm body weight	37 [2]	43	53	44
	Adrenal ascorbic acid, mg %	41	23	20	28
	Urinary ascorbic acid, μg per day [1]	26	37	26	30
	Odontoblast height, μ	.. [3]	.. [3]	.. [3]	..
Averages of columns	Adrenal weight, mg per 100 gm body weight	47	58	68	
	Adrenal ascorbic acid, mg %	51	40	31	
	Urinary ascorbic acid, μg per day [1]	12	15	12	
	Odontoblast height, μ	..	54	38	

[1] During the last week of the 7-week experiment.

[2] Animal died of hernia. Value calculated by method shown in table 3.

[3] Height depended on area of tooth observed.

disorganized, broken, and only about one-half as high as those formed in the early stages of the test. The odontoblasts of the later period are not comparable to those of the saline-injected controls receiving the same amount of ascorbic acid. The data were not subjected to statistical analysis due to inability to assign a definite value to the animals which had been treated with 5 mg of cortisone. Visual inspection indicated little difference between the animals receiving a 2.5-mg level of cortisone or saline (these values are similar to those obtained in untreated male animals of comparable weights), indicating that the 2.5-mg level of cortisone had probably not changed the requirement for ascorbic acid. The 5-mg level of cortisone, while initially sparing ascorbic acid, was not enough to prevent certain changes in the odontoblasts of guinea pigs which remained on the experiment 56 days. Sections of the incisor of animal 378, which received 5 mg of cortisone per day and 0.40 mg of ascorbic acid per 100 gm body weight, are shown in plate 1.

DISCUSSION

The results from the experiments using endocrine-active materials are probably not subject to a simple interpretation. In an extensive series of investigations on guinea pigs, Silberberg and Silberberg ('38, '39, '40, '41, '48) have shown that the level of treatment used is very critical and the response may be entirely reversed by a slight change in dosage level. Silberberg and Silberberg ('38) reported that desiccated thyroid was more stimulatory in female than in male guinea pigs.

The fact that symptoms of gross scurvy appeared in some animals receiving iodinated casein and amounts of ascorbic acid about twice that which protects untreated animals from scurvy is good evidence in support of the thesis of an increase in the requirement. No similar manifestations were noted in the experiments with cortisone or thiouracil.

It should be emphasized that effects produced during short 15- to 21-day experiments with cortisone may be only

temporary. On the basis of the second week's growth, the cortisone-treated animals would have been judged to be giving the best response obtained in any of the tests. On the basis of the odontoblasts formed during the initial days on the experiment, the cortisone-treated animals were showing a lowered requirement for ascorbic acid. By the end of the 56-day period, the saline-injected controls had gained more weight and had odontoblasts more nearly approaching those of positive control animals than did those animals receiving 5.0 mg cortisone.

That ascorbic acid may be involved in cortisone formation is indicated by the *in vitro* work of Seneca et al. ('50). The appearance of ascorbic acid in the urine and the decreased adrenal storage may be explained if one assumes that ascorbic acid is taken up by the adrenal gland in anticipation of a future need for the elaboration of cortical hormones. If one supplies one of the cortical hormones and, therefore, slows the production of adrenal corticotropic hormone (ACTH), the cortex is no longer stimulated to take up ascorbic acid and there is then a urinary spill-over. The work of Hyman et al. ('50) indicates that in scurvy the adrenals hypertrophy under the influence of ACTH. They interpret this to mean that ascorbic acid is not involved in the production of cortisone and indicate that since ACTH prolonged the life of scorbutic animals, there is no greater need for ascorbic acid under conditions of adrenal activity than when the adrenals were quiescent (animals were given cortisone). However, they did not kill the animals from the different groups at comparable times and the results might have been different if that had been done.

It will be noted from tables 3 and 4 that ascorbic acid concentrations of less than 63 mg % are frequently associated with adrenal glands of normal size. Pfander and Mitchell ('52) estimated that 63 mg % of ascorbic acid in the adrenal glands was necessary to prevent hypertrophy as well as impairment of odontoblast growth. It may be concluded that

probably both thiouracil and cortisone are involved in the ascorbic acid economy of the adrenal glands.

CONCLUSIONS

1. The administration of a level of iodinated casein sufficient to produce a 25% increase in heart rate increases the ascorbic acid requirement of the male guinea pig about 45%.
2. A level of thiouracil of 60 mg per kilogram body weight does not appreciably affect the ascorbic acid requirement.
3. The only effects of 2.5 mg of cortisone per day are (a) a decrease in adrenal size and (b) a slight rise in urinary ascorbic acid. Five milligrams of cortisone appear to have an initial sparing action on the ascorbic acid requirement, but by the end of 56 days the effect disappears and there is evidence of retrogressive changes in the odontoblasts.
4. Cortisone causes a rise in the urinary excretion of ascorbic acid associated with an average decrease in the concentration of ascorbic acid in the adrenal glands of doubtful statistical significance.
5. It should be emphasized that the favorable initial effects of cortisone administration may be followed by a period in which the growth of the animal becomes very slow, and the odontoblasts cease to maintain an organized state. Caution is therefore suggested in making recommendations based on short-term experiments with cortisone.

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PLATE 1

EXPLANATION OF FIGURES

Microphotographs of representative histological sections of the incisor teeth of experimental guinea pigs. Magnification approximately 860.

1 Hyperthyroid — 0.70 mg ascorbic acid per 100 gm body weight.

2 Hyperthyroid — 0.55 mg ascorbic acid per 100 gm body weight.

3 Hyperthyroid — 0.40 mg ascorbic acid per 100 gm body weight.

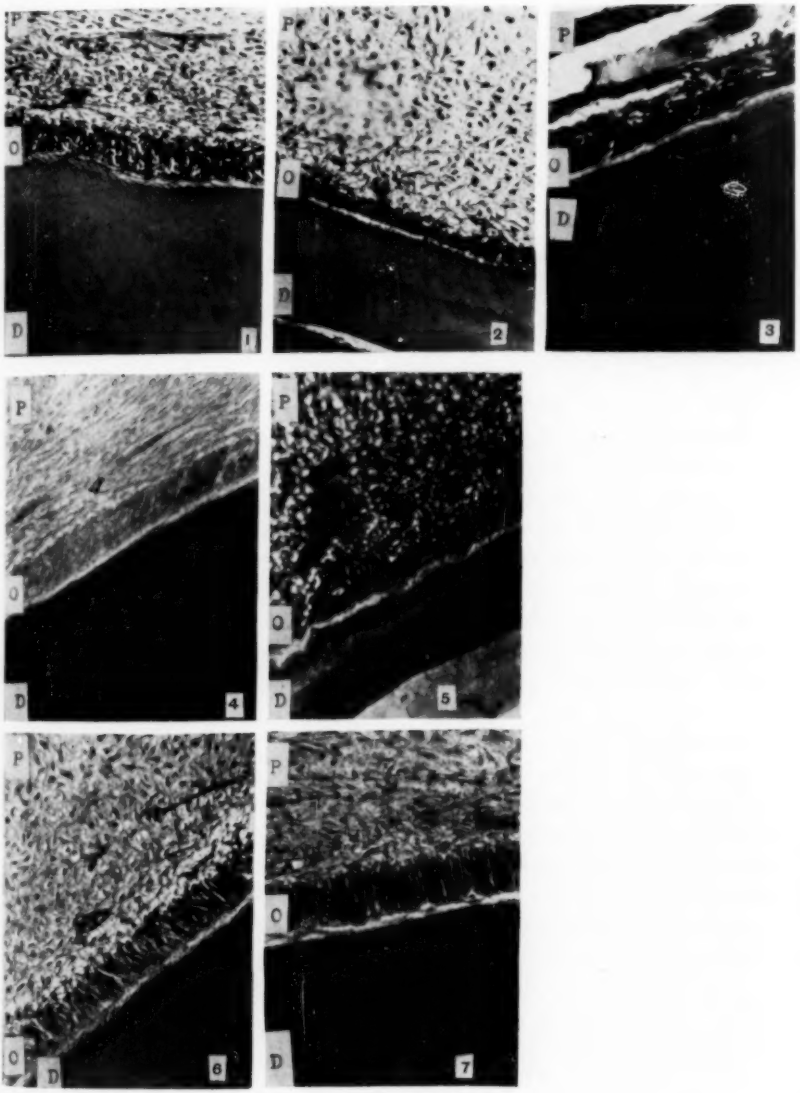
4 Five milligrams cortisone per day — 0.40 mg ascorbic acid per 100 gm body weight. These odontoblasts were formed in the early stages of the experiment.

5 Same tooth as 4, showing odontoblasts formed after 4 weeks on the experiment.

6 Sixty milligrams thiouracil per kilogram body weight — 0.70 mg ascorbic acid per 100 gm body weight.

7 Sixty milligrams thiouracil per kilogram body weight — 0.55 mg ascorbic acid per 100 gm body weight.

P = pulp
O = odontoblasts
D = dentine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THE ASCORBIC ACID REQUIREMENT OF THE GUINEA PIG WHEN ADRENAL WEIGHT AND ODONTOBLAST HEIGHT ARE USED AS CRITERIA[1]

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FIVE FIGURES

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In order to maintain the metabolic and structural integrity of the several tissues and organs, a certain amount of ascorbic acid is needed. The amount of ascorbic acid required for these several needs, plus any that may be destroyed by the action of intestinal microorganisms, minus any that may be supplied by tissue synthesis, is the amount of ascorbic acid which the guinea pig must obtain from exogenous sources. This may be considered as the daily requirement. As a measure of the protection afforded by a given amount of ascorbic acid, many criteria have been used.

One of the early methods was that of Höjer ('26), in which over-all protection to the incisor teeth was the criterion. Dann and Cowgill ('35) used this method to establish the requirement of the guinea pig for vitamin C when lemon juice was the source material; they related the requirement to body weight. This method was refined by Boyle et al. ('40), who studied the dentine layers, and was more recently put on a quantitative basis by Crampton ('47), who measured the height of the odontoblast cells.

[1] The data reported in this paper are taken from a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

[2] Fulbright Fellow now at Rowett Research Institute, Aberdeen, Scotland.

McCarrison ('19) observed that the adrenal glands of guinea pigs on an oat diet increased in size. Histological examination indicated that the increase in size was due to a stasis of the venous system and enlargement of the cortical layer.

LaMer and Campbell ('20) reported that the weight of the adrenals in scorbutic guinea pigs was double that in healthy animals. Dugal and Thérien ('49) were able to prevent the increase in adrenal size which occurred in a cold environment by giving 10 mg of ascorbic acid daily to the guinea pigs while they were in the cold room (2°C. for 40 days).

The hypothesis has been advanced by Pijoan and Lozner ('44) that the white blood cells in man cling tenaciously to their stores of ascorbic acid until the plasma stores are exhausted. Lowry et al. ('46) obtained data on human subjects showing that the variation in ascorbic acid in the white blood cells of subjects on different levels of the vitamin very roughly parallels the variation in serum ascorbic acid concentration. In the guinea pig the level of ascorbic acid in the whole blood is related to tissue concentration and, at inadequate levels of ascorbic acid, to histological changes in the incisor teeth (Kuether, Telford and Roe, '44).

This investigation was undertaken with the object of determining the daily oral dose of ascorbic acid in aqueous solution that would keep the test animals in a physiological condition in respect to the criteria studied, comparable to that of animals of similar weight and age on the same diet supplied amounts of the vitamin two to three times that of the test doses. The requirement is expressed in relation to average body weight at the end of the experimental period.

EXPERIMENTAL

After the animals were received from a local dealer, they were weighed and placed on the scorbutogenic experimental diet for two weeks, during which time they were fed ad libitum and the daily food intake and bi-weekly weights were recorded. In the first two days they received 250 mg of ascorbic acid in

an effort to bring all animals into an initial stage of saturation. At the end of a two-week depletion period the animals were divided into pairs or trios on the basis of previous food intake, body weight, and genetic background, if known.

The diet used in these studies was modified from that used by Crampton ('47).[3] It was found to be free of ascorbic acid by the xylene extraction method of assay (Robinson and Stotz, '45) and by the fact that negative controls died of scurvy in 20 to 25 days. The diet contained 25% crude protein (N X 6.25) and was adequately fortified with minerals and vitamins other than ascorbic acid. It was fed in pelleted form.

The guinea pigs were kept in individual cages in an air-conditioned room at approximately 75°F.; during extremely hot weather the air-conditioner was unable to prevent some rise in room temperature. Water in 8-ounce bottles with drinking tubes was kept before the animals at all times. Water intake was recorded daily in one series of experiments.

The ascorbic acid solution was prepared by weighing 200 mg of ascorbic acid on an analytical balance and transferring them to a 50-ml volumetric flask; the contents were then made up to 50 ml with distilled water. The guinea pigs were given the selected dose in proportion to body weight by a pipette graduated to 0.01 ml. Ascorbic acid was always given in the morning before feeding and the order of administration was changed among the animals of a group at three-day intervals throughout the experiment. On two occasions the ascorbic acid content of the solution after all animals had been dosed was compared to a similar amount mixed with metaphosphoric acid. There had been no detectable loss of ascorbic acid from the aqueous solution. The dosages of ascorbic acid varied in the different experiments. In all experiments at least one negative control and one positive control were run. The positive controls received amounts of

[3] The modifications consisted of changing the proportions of some of the ingredients, substituting linseed oil meal for soybean oil meal and adding a supplement of vitamins of the B complex.

ascorbic acid 20 to 100% greater than those of animals on the highest test dose.

Animals were weighed to the nearest 10 gm twice a week. The dosage of ascorbic acid for the subsequent three- or 4-day period was based on the body weight of the guinea pigs observed on these days.

Food was weighed daily. If refusals had occurred and the food still appeared wholesome, the allowance for all animals in the trio was reduced. If the food was contaminated, it was air-dried, weighed and discarded, and adjustments were then made on later days. Controls were fed ad libitum and the food intake measured.

In one experiment, animals were placed in glass metabolism cages and 24-hour urine samples were collected into metaphosphoric acid.

After being on the experiment for 7 weeks, the animals were fasted for 24 hours, the last 4 without water. The order of killing and of the withdrawal of food and water was determined at random. Animals were sacrificed by a blow on the head; 5 ml of blood were drawn by heart puncture and transferred to a bottle containing double oxalate (Wintrobe, '46) and stored at 34°F. until ready for analysis. The adrenal glands were removed, trimmed free of connective tissue and blotted free of blood and weighed to the nearest 0.1 mg. The adrenals to be used for ascorbic acid analysis were transferred to a bottle containing 5 ml of 5% metaphosphoric acid and stored at 34°F. until ready for analysis. The adrenals for histological study were transferred to either formaldehyde-saline or acidified silver nitrate solutions, then thiosulfite and finally 70% ethyl alcohol (Barnett and Bourne, '41). The adrenals for proximate analysis were placed in air-tight containers and frozen at 0°F.

The lower jaws of the guinea pigs were removed and trimmed, wrapped in gauze, labeled, and placed in 8% formaldehyde in a 0.9% NaCl solution.

The contents of the gastrointestinal tract and urinary bladder were removed and the carcasses were examined for gross

pathology. If gross changes in any of the organs were observed, portions were fixed in formaldehyde-saline or Ladowinski's FAA (formaldehyde 2, 85% ethyl alcohol 17, and glacial acetic acid 1). Trios which contained animals showing gross pathology were excluded in the analysis of the data.

The remainder of the carcass was wrapped in heavy brown paper and stored at 0°F. After some practice, the entire process of collecting the various tissues could be completed in less than 20 minutes.

Within 12 hours after the termination of an experiment, the hemoglobin content of the blood was estimated by the method of Evelyn ('36). Four milliliters of the blood were fractionated into plasma, red and white cells, and the ascorbic acid content of the plasma and white cells was estimated by the method of Roe and Kuether as modified by Lubschez ('45). In general, the method for determining adrenal ascorbic acid was that of Roe and Kuether ('43).

The nitrogen content of some of the carcasses was determined by the modified Kjeldahl-Gunning-Arnold procedure (Association of Official Agricultural Chemists, '50). The carcasses were taken from the 0°F. room, weighed, and ground in the frozen state through a Hobart mill. The sample was stored at 0°F. until ready for analysis.

Crampton ('47), in his article on the odontoblast procedure, gave the treatments which he used for paraffin embedding. In the preliminary work these were tried, but since some shrinkage was observed, certain changes were made. Essential changes were: (1) fixation in 8% formaldehyde in saline instead of 10% formaldehyde; (2) the reduction of the nitric acid concentration from 10 to 5% for decalcification; (3) the use of a chemical test, sodium oxalate in ammonium hydroxide (Morse, '45), instead of needling the teeth, to determine when decalcification was complete; (4) regressive staining and the use of the connective tissue stain of Mulligan ('46). Some of the jaws were embedded in celloidin. This procedure was modified from that of Walls ('32).

Adrenals for histological study and tissues which showed gross pathology were dehydrated through alcohol, cleared in xylol and embedded in paraffin. They were sectioned at 10 μ and stained with Harris hematoxylin and eosin or with triple chrome (Mulligan, '46).

To study the effects of sex, 10 trios of female guinea pigs were studied using the procedures described above for male guinea pigs. Five trios were comparable to the males run concurrently and 5 were comparable to the males used in an experiment studying the effects of hyperthyroidism.

RESULTS

The male guinea pig

There was no tendency for animals receiving 0.40 mg of ascorbic acid per 100 gm body weight to refuse feed more frequently than those receiving 0.70 mg per 100 gm. (The Chi square test gave a P value > 0.1.)

Analysis of variance of the pertinent data showed that increase in body weight over the experimental period was independent of the dosage of ascorbic acid if as much as 0.30 mg per 100 gm body weight was supplied ($F < 1$). The nitrogen in the carcass was also independent of the test levels within the narrow range used ($F < 1$).

The hemoglobin values were lower in some of the animals than the normal range of 13 to 14.5 gm % cited by Wintrobe ('46) but the test levels of ascorbic acid had no influence on the levels of hemoglobin.

In the one experiment where measurements were made, the animals receiving 0.40 mg ascorbic acid per 100 gm body weight consumed no more water than those receiving 1.4 mg per 100 gm body weight ($P > 0.2$).

No ascorbic acid was found in the urine of guinea pigs supplied 0.7 mg (or less) of ascorbic acid per 100 gm body weight.

In general, mean values for blood and leukocyte concentration of ascorbic acid reflected the intake, but the variation

for any one dose level was so great as to preclude the use of these measurements as criteria of nutritive adequacy.

Due to certain mechanical difficulties, the blood plasma ascorbic acid levels are incomplete; however, generally the plasma and white cells contain some ascorbic acid even when the adrenals have hypertrophied and the odontoblasts have become disarranged. The difficulty inherent in the use of blood levels of ascorbic acid to assess vitamin C nutriture has been reviewed by Karel and Chapman ('44), who present evidence showing that "In the determination of the state

TABLE 1

Ascorbic acid concentration in adrenal glands at different ascorbic acid intakes: male guinea pigs

ASCORBIC ACID INTAKE IN MG/100 GM BODY WEIGHT	NUMBER OF GUINEA PIGS	AVERAGE ASCORBIC ACID IN ADRENAL GLANDS IN MG %, WITH STANDARD ERROR		COEFFICIENT OF VARIATION, %
		Observed	Estimated [1]	
0.70	8	74.7 ± 5.1	76.0	18.2
0.55	19	58.5 ± 3.7	57.9	27.1
0.40	19	43.3 ± 2.7	39.8	26.7
0.30	11	25.0 ± 3.9	27.8	44.8

[1] From the regression equation: $y = -8.34 + 120.46x$, in which y is the concentration of ascorbic acid in the adrenals in milligrams per cent, and $\times$ is the dosage of the vitamin in milligrams per 100 gm body weight.

of vitamin C saturation or depletion of guinea pigs, the individual variation and the time elapsing between the last intake of the vitamin and the withdrawal of the blood sample from the animal should be considered." In these experiments the individual variability of guinea pigs in this respect is represented by a coefficient of variation of 54%.

The ascorbic acid concentration in the adrenal glands of the male pigs (y) varied directly with the dosage of ascorbic acid, as the values in table 1 reveal. The relationship is evidently a linear one within the limits of dosage studied, and the agreement between observed values of y for the different ascorbic acid dosages (x) and the values computed from the

regression equation is good. The negative constant, — 8.34, if significantly different from zero, would mean that at lower levels of ascorbic acid intake the regression departs from linearity. The correlation of the concentration of ascorbic acid in the adrenal glands with the weight of adrenals is — 0.663 for 64 paired observations. A coefficient of 0.306 for 68 paired variates establishes a correlation with a probability of 0.01 (Fisher, '44).

TABLE 2

The differences in adrenal weight per 100 gm body weight and odontoblast height between different ascorbic acid dosages

ASCORBIC ACID DOSAGES IN MG/100 GM BODY WEIGHT DAILY, COMPARED	AVERAGE DIFFERENCE IN ADRENAL WEIGHT PER 100 GM BODY WEIGHT			AVERAGE DIFFERENCE IN ODONTOBLAST HEIGHT		
	N	Average [1]	P	N	Average [2]	P
		mg			*μ*	
Male guinea pigs						
0.70 vs. 0.55	18	8.61	0.052	18	10.11	0.0042
0.55 vs. 0.40	20	8.70	0.009	20	17.15	0.0001
Female guinea pigs						
0.70 vs. 0.55	8	6.25	0.096	8	8.50	0.0006
0.55 vs. 0.40	7	2.14	0.32	7	10.14	0.039

[1] These averages all favor the lower of the two doses compared, in the sense that the lower dose is always associated with the larger adrenal weight per 100 gm body weight.

[2] These averages all favor the larger of the two doses compared, in the sense that the higher dose is always associated with the greater odontoblast height.

The male guinea pigs receiving 0.70 mg of ascorbic acid per 100 gm body weight per day showed significantly higher odontoblast cells than those receiving smaller doses (table 2). It can be seen, furthermore, from plate 1 that the odontoblasts from the incisors of the positive controls, receiving 1.40 mg of ascorbic acid per 100 gm body weight per day, were quite similar in appearance and height to those from guinea pigs receiving 0.70 mg.

The average odontoblast heights with their standard errors for 9 control male guinea pigs receiving 0.85 or 1.40 mg of

ascorbic acid per 100 gm body weight daily, and for 20 guinea pigs receiving 0.70 mg, are 62.1 ± 1.2 and $59.5 \pm 1.6\,\mu$, respectively. The difference, $2.6 \pm 2.0\,\mu$, is evidently not significant. The probability of significance by Fisher's "t" test is about 0.3.

It may, therefore, be concluded that the requirement of the male guinea pig for ascorbic acid, as measured by this sensitive test, is 0.70 mg per 100 gm body weight, or somewhat less.

As measured by the adrenal weight per 100 gm body weight, also, the 0.70-mg dose of ascorbic acid is probably ($P = 0.052$) superior to the 0.55-mg dose (see table 2), and the latter dose is definitely ($P = 0.009$) more effective than the 0.40-mg dose in preventing enlargement of the adrenal glands in the male guinea pig.

The average weight of the adrenal glands of the 20 male guinea pigs that received 0.70 mg ascorbic acid daily per 100 gm body weight, with its standard error, was 62.1 ± 3.9 mg; that of control animals, 5 of which received doses of 1.4 mg and 4 of 0.85 mg per 100 gm body weight daily, was 58.1 ± 2.1. The difference of 4.0 ± 4.4 is obviously not significant. Analysis of the two groups by means of the Fisher "t" test gives a probability of 0.8 that a difference as large as this might have resulted from the fortuitous combination of the uncontrolled factors in the experiment. It may, therefore, be concluded that the ascorbic acid requirement of growing male guinea pigs, within the weight ranges studied, is approximately 0.70 mg daily per 100 gm body weight. This criterion of nutritional adequacy with reference to ascorbic acid requirement, therefore, yields essentially the same result as that based on odontoblast height in the incisor teeth.

In an attempt to measure in male guinea pigs the concentration of ascorbic acid in the adrenal glands associated with incipient scurvy, the regressions of this concentration in milligrams per cent (y) on adrenal weight in milligrams per 100 gm body weight (x) and odontoblast height in microns

(z) were determined (regardless of ascorbic acid dosage) by the method of least squares, with the following results:

$$y = 105.85 - 0.7404x,\ r_{x \cdot y} = -0.670,\ n = 57 \quad (1)$$

$$y = 27.64 + 0.6382z,\ r_{y \cdot z} = +0.848,\ n = 37 \quad (2)$$

Both the coefficients of regression and of correlation are significant at probability levels of less than 1%. The values of x and z corresponding to the dosage of ascorbic acid estimated to be at, or very near, the requirement, i.e., 0.70 mg per day per 100 gm body weight, are 62.1 mg and 59.5 μ, respectively (see above). Substituting these values in the above equations gives a value of 59.8 mg % from equation (1), and 65.6 mg % from equation (2). These values are not distinguishable statistically ($t = 0.86$, $P = 0.4$). Hence, it may be concluded that the best estimate, from the data of these experiments, of the concentration of ascorbic acid in the adrenal glands associated with incipient scurvy is about 63 mg %.

The female guinea pig

The ascorbic acid requirement of the female guinea pig was studied in two groups of 5 trios each. Five trios were studied in the spring and 5 trios in the summer of 1950. The two groups were of different genetic background.

The values for blood and adrenal concentrations of ascorbic acid, for hemoglobin concentration, and for food consumption in the females were similar to those of the males in a comparable stage of development.

Referring again to the average experimental results presented in table 2, it is apparent that, with the female guinea pigs as with the male, the 0.70-mg dosage of ascorbic acid per 100 gm body weight was definitely superior to the 0.55-mg dosage in maintaining the height of the odontoblast cells of the incisor teeth, while the 0.55-mg dosage was better than the 0.40-mg dosage at the 4% probability level. The average height of the odontoblast cells at the highest ascorbic acid dosage was 53.2 ± 2.4 μ (9 cases) for the females as compared with 59.5 ± 1.6 μ (20 cases) for the male guinea pigs.

The difference of $6.3 \pm 2.9\,\mu$ is on the borderline of significance ($P = 0.04$). Unfortunately no positive controls were run for the female guinea pigs.

The weight of the adrenals of the female guinea pigs per 100 gm body weight was not significantly different between adjacent dosages of ascorbic acid, although the averages were consistently inversely correlated with these dosages.

The validity of body weight as a reference for ascorbic acid requirements

In these experiments the dosages of ascorbic acid were given in proportion to body weight on the basis of the evidence of Dann and Cowgill ('35) that the need of the guinea pig for this vitamin is in proportion to body weight. Confirmation of this conclusion is contained in tables 3, 4 and 5.

In table 3 it is clear that there is no tendency for the odontoblast height associated with the different dosages of ascorbic acid either to decrease or to increase with the body weight of male guinea pigs. If the requirement for ascorbic acid were not related directly to body weight, trends in either one or the other direction would be expected because, especially for the lower dosages per 100 gm body weight, the odontoblast height would be expected to be less at the higher weights if the requirement were greater at these weights, or to be greater if the requirement were less.

Table 4 reveals the same situation with reference to adrenal weight per 100 gm body weight.

In table 5, the correlation coefficients of odontoblast height and of adrenal weight per 100 gm body weight with body weight are summarized for each ascorbic acid dosage. For the number of paired variates available, none of these coefficients is significant at the 10% probability level, except the coefficient between odontoblast height and body weight at the 0.55-mg dosage of ascorbic acid, which is significant at a probability level of less than 2% (Fisher, '44).

Certainly the preponderance of evidence favors the conclusion that there is no appreciable effect of body weight on

TABLE 3

The relation of body weight to odontoblast height at different ascorbic acid dosages for male guinea pigs

NUMBER OF TRIOS INVOLVED [1]	RANGE IN AVERAGE FINAL BODY WEIGHTS	ASCORBIC ACID DOSAGE IN MG PER 100 GM BODY WEIGHT			STATISTICAL ANALYSIS OF DIFFERENCES [2]	
		A = 0.7	B = 0.55	C = 0.40	(A-B) P =	(B-C) P =
		Average odontoblast height				
	gm	*μ*	*μ*	*μ*		
2	< 300	57	54	44	..	..
8	300–400	61.6	39.2	24.0	0.0015	0.0016
10	400–500	52.9	50.3	30.0	0.32	0.0007
4	500–600	53	53	47	..	..
2	600–700	..	62	37	..	..
0	700–800	..	..	..	..	..
2	800–900	64	59	..	..	..

[1] The data for all the trios are not complete.
[2] By Fisher's ('44) "t" value method.

TABLE 4

The relation of body weight to adrenal weight per 100 gm body weight at different ascorbic acid dosages: male guinea pigs

NUMBER OF TRIOS INVOLVED [1]	RANGE IN AVERAGE FINAL BODY WEIGHTS	ASCORBIC ACID DOSAGE IN MG PER 100 GM BODY WEIGHT			STATISTICAL ANALYSIS OF DIFFERENCES [2]	
		A = 0.70	B = 0.55	C = 0.40	(B-A) P =	(C-B) P =
		Average adrenal weight per 100 gm final body weight				
	gm	*mg*	*mg*	*mg*		
2	< 300	61	62	73	..	..
8	300–400	70	75	84	0.26	0.15
10	400–500	58	70	77	0.11	0.026
4	500–600	51	69	69		
2	600–700	..	62	70		
3	700–800	..	72	88	0.012	0.0021
5	800–900	61	74	85		

[1] The data for all the trios are not complete.
[2] By Fisher's ('44) "t" value method.

the requirement of the male guinea pig for ascorbic acid per unit of body weight. The conclusion seems justified, therefore, that the daily requirement of the male guinea pig for ascorbic acid (C) may be estimated by the following equation:

$$C = 0.70W,$$

in which C is expressed in milligrams and the body weight (W) in hundreds of grams.

The absolute adrenal weight is highly correlated with body weight to the extent that $r = +0.801$ ($N = 38$), combining the results secured with the 0.55-mg and the 0.40-mg doses of ascorbic acid per 100 gm body weight. It is also correlated

TABLE 5

Correlation of adrenal weight per 100 gm body weight, and odontoblast height, with final body weight in male guinea pigs

ASCORBIC ACID DOSAGE	CORRELATION WITH FINAL BODY WEIGHT [1]			
	Adrenal weight		Odontoblast height	
	N	R	N	R
mg				
0.70	22	− 0.315	22	+ 0.0093
0.55	33	+ 0.297	24	+ 0.472
0.40	32	+ 0.151	23	+ 0.234

[1] N = number of paired variates; R = product — moment correlation.

with body nitrogen, with a correlation coefficient of + 0.538, using the same trios for both correlations. The difference between these two correlations is significant at the 5% level when tested by Fisher's method after transformation of the r's to z's. Hence, body weight has been shown to be probably a better reference than body nitrogen in the expression of ascorbic acid requirement in the guinea pig. The nitrogen determinations on guinea pigs receiving 0.70 mg of ascorbic acid per 100 gm body weight were too few (8 cases only) to make a correlation study a profitable undertaking. For the same reason, a correlation study of the data for the female guinea pigs has not been made. The nitrogen content of the female guinea pigs analyzed was appreciably lower than that

of the males, averaging $3.04 \pm 0.01\%$ as compared with $3.42 \pm 0.01\%$ for the males, probably reflecting the higher fat content of the female animal.

The relative sensitivity of the two criteria of ascorbic acid adequacy used

The two criteria of ascorbic acid adequacy employed in these experiments, i.e., the minimum dosage of the vitamin required (1) to prevent adrenal gland hypertrophy, and (2) to maintain normal odontoblast cells in the incisor teeth, gave essentially the same result, 0.70 mg of ascorbic acid per 100 gm of body weight per day for male guinea pigs weighing initially from 110 to 840 gm. This result is in good agreement with that of Dann and Cowgill ('35), who reported a requirement of 1 ml of lemon juice (0.60 mg of ascorbic acid per milliliter, according to Rosenberg, '45) per 100 gm body weight per day, which may not be significantly less than the value found in the investigation reported in this paper. The failure to detect any difference in the requirement between male and female guinea pigs is in agreement with Crampton's ('47) observation that males and females may be used interchangeably for the assay of ascorbic acid.

However, the correlation of adrenal weight per 100 gm body weight and odontoblast height, pooling the results for all ascorbic acid dosages to give 70 paired variates, is not high, — 0.499. This means that only 25% (r^2) of the variation in the two measurements is accounted for by the correlation between them, leaving 75% unaccounted for.

Of the two criteria, the odontoblast height is evidently the more sensitive. In all 4 comparisons given in table 2, the smaller probability of a fortuitous outcome is given by the odontoblast height. In fact, only this method revealed significant differences between adjacent dosages of ascorbic acid for the female guinea pigs. Furthermore, the correlation of odontoblast height with the ascorbic acid concentration in the adrenal glands is greater at the 5% probability level than

the correlation of adrenal weight per 100 gm body weight with the ascorbic acid content of the adrenal glands.

The greater sensitivity of the odontoblast height over the adrenal weight as a criterion of ascorbic acid nutriture is not a matter for surprise, since it is probably more specific: several types of stresses will cause an increase in adrenal size (Selye, '50). The sole advantages of the adrenal weight criterion are the shorter time probably required to obtain the result of a test, and the employment of a simple gravimetric technic. It has been reported that guinea pigs with gross or clinical signs of scurvy show marked enlargement of the adrenals after 21 days on a scorbutogenic diet (Bessesen, '23; Stepto et al., '51), and that the size of the adrenal gland depends on the length of time the animal is kept on the deficient diet (LaMer and Campbell, '20). On the basis of the observations of these workers, it would seem that measurable differences in adrenal weight would be evident between marginal and adequate diets after 28 to 35 days.

DISCUSSION

This investigation has confirmed the earlier work of Dann and Cowgill ('35) in establishing a direct relationship in the guinea pig between the ascorbic acid requirement and body weight. The requirement of only one other nutrient, i.e., vitamin A (Guilbert, Howell and Hart, '40), has been shown to vary directly with body weight. The relationship for vitamin A has been shown to apply for species of animals varying in size from the rat to the horse and the ox, thus possessing as great an inter-specific validity as it does an intra-specific validity. In particular it has been shown to apply to the human infant (Lewis and Haig, '39) and to the human adult (Booher, Callison and Hewston, '39).

On the basis of these findings with reference to vitamin A, one might expect the ascorbic acid requirement of the guinea pig and of man to be approximately the same per unit of body weight. But this is not the case. The guinea

pig requires 7 mg of ascorbic acid per kilogram of body weight. The requirement for man has not been precisely determined, but whether the value of 10 mg daily for an average man (Medical Research Council, '48) or the much larger value of 75 mg required for the prevention of gingivitis according to Linghorne et al. ('46) is taken, the requirement per kilogram of body weight, 0.14 or 1.07 mg, respectively, is much less than the analogous value for the guinea pig.

There is also a great disparity in the requirements when expressed on the surface area basis. A 500-gm guinea pig has a surface area of 0.0526m² (Kibler, Brody and Worstell, '47) and an ascorbic acid requirement of 67 mg per m² per day. A 70-kg man with a surface area of 1.8m² would require from 5.6 to 42 mg per m² per day, depending upon which estimate of the requirement is used in the computation.

A possible explanation of the relatively higher requirement of ascorbic acid by the guinea pig is the presence in man of a mechanism for synthesizing the vitamin but at a rate inadequate to cover the requirements, or a mechanism for secreting some substance which spares ascorbic acid.

SUMMARY AND CONCLUSIONS

A study has been made of the ascorbic acid requirements of the guinea pig in relation to body weight, body nitrogen and sex. Trios of guinea pigs were fed doses of ascorbic acid ranging from 0.30 to 0.70 mg per 100 gm body weight; other animals served as positive and negative controls. Within each trio the consumption of the basal diet free of ascorbic acid was equalized throughout the feeding period of 7 weeks. The adequacy of the various doses of ascorbic acid was judged by the height of the odontoblast cells of the incisor teeth and by the weight of the adrenal glands per unit of body weight. The results obtained support the following conclusions:

1. By both criteria, the daily ascorbic acid requirement of the male guinea pig between the weights of 110 and 840 gm was found to be approximately 0.70 mg per 100 gm body

weight. No sex difference in this requirement was demonstrated.

2. The requirement varies directly with body weight; the correlation with body nitrogen is less close.

3. The odontoblast height of the incisor teeth is a much more sensitive criterion of ascorbic acid nutriture than is the weight of the adrenal glands per unit of body weight. The latter criterion, however, is serviceable and simpler.

4. The ascorbic acid concentration of the adrenal gland is correlated with the dosage of the vitamin, as well as with the adrenal weight and the odontoblast height. From the regression equation of ascorbic acid concentration of the adrenal glands on adrenal weight and on odontoblast height, it was computed that a concentration of 63 mg % is associated with incipient enlargement of the adrenal glands and incipient changes in the odontoblast cells of the incisor teeth.

5. The guinea pig does not excrete ascorbic acid in the urine when receiving as much as 0.70 mg per 100 gm body weight.

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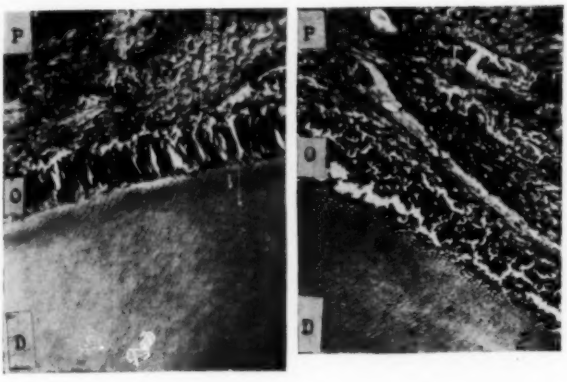
PLATE 1

EXPLANATION OF FIGURES

Microphotographs of representative histological sections of the incisor teeth of experimental guinea pigs. Magnification approximately 860.

1 Positive control pig.
2 Scorbutic guinea pig.
3 Given 0.70 mg ascorbic acid per 100 gm body weight.
4 Given 0.55 mg ascorbic acid per 100 gm body weight.
5 Given 0.40 mg ascorbic acid per 100 gm body weight.

O = odontoblasts
P = pulp
D = dentine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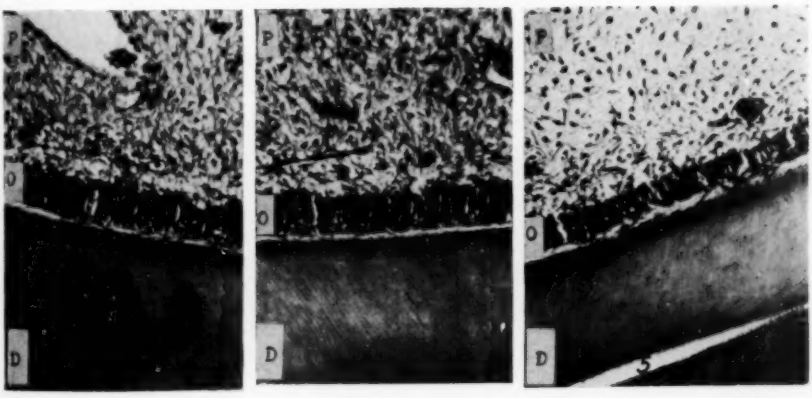
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THE URINARY EXCRETION OF VITAMIN B_{12} FOLLOWING SUBCUTANEOUS ADMINISTRATION TO YOUNG AND ADULT RATS WITH DIFFERENT RESERVES [1]

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ONE FIGURE

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Either oral or parenteral administration of vitamin B_{12} will stimulate the rate of growth of the offspring of rats fed a soybean meal diet (Emerson et al., '49) but is without effect on the growth rate of the progeny of rats (Hartman et al., '49) fed diets containing milk proteins and liver. This difference may be explained if weanlings from milk protein- and liver-fed parents, in contrast to those from soybean meal-fed parents, have reserves of vitamin B_{12} adequate to meet their growth requirements. Assuming the validity of this hypothesis, the offspring of rats fed the soybean meal diet may be called deficient in B_{12} reserves; the others, normal. It is, therefore, of interest to compare the rates of excretion of vitamin B_{12} following its subcutaneous injection in both types of animals. Data for making such comparisons have been obtained by studying the growth rates, food consumption and urinary excretion of Co^{60} of groups of young weanling rats of each type, and of adult rats of

[1] The authors wish to acknowledge grants-in-aid from the Squibb Institute for Medical Research, Sharp and Dohme, Inc., Upjohn and Company, Merck and Company, and the National Vitamin Foundation.

different states of B_{12} sufficiency, following subcutaneous injection of a vitamin preparation containing Co^{60}.

EXPERIMENTAL

1. Radioactive substances

A solution of vitamin B_{12} containing Co^{60} was kindly supplied by a commercial firm.[2] The dissolved vitamin was as pure as the crystalline compound in terms of its microbiological activity per milligram; the activity was 60 microcuries per milligram of vitamin B_{12} (Rosenblum and Woodbury, '51). In another part of the experiment, Co^{60} in the form of cobalt chloride with specific activity of one microcurie/milligram,[3] was used.

2. Preparation of animals

For the purpose of this report, animals are described as deficient, normal or saturated. These terms are defined by the histories of the animals as follows:

Deficient animals are those born of stock colony adults which were transferred to a basal soybean ration at the time of mating and maintained on that ration until the young were weaned. Some of these weanlings were used at that time; others were continued on the soybean ration to maturity.

Normal animals are stock colony animals born of parents fed a milk protein- (8%) and liver (2%)-containing diet during their lives. The young were either used as weanlings or maintained on this stock diet[4] until introduced into an experimental group.

[2] Merck and Company. This vitamin preparation is designated hereafter as vitamin B_{12}^{*}.

[3] Obtained from the Oak Ridge Laboratories with the permission of the Atomic Energy Commission.

[4] Stock diet: whole wheat (ground), 30 lb.; maize (ground), 20 lb.; rolled oats (ground), 18 lb.; whole milk powder (Kraft), 12½ lb.; skimmed milk powder (Kraft), 12½; Casal (mixture of casein and lactalbumin; Crest Food Co., Ashton, Illinois), 4 lb.; liver powder (Wilson), 2 lb.; NaCl, ½ lb.; $CaCO_3$, 120 gm; Fe citrate, 50 gm; and $CuSO_4$, 20 gm.

Saturated animals are normal animals offered during the two weeks preceding an experiment the stock ration (10 gm) containing 2.5 μg of vitamin B_{12} per day and given subcutaneously, during the week preceding the experiment, 5 μg of vitamin B_{12} daily.

3. Diet

The composition of the diet used during the experiment was as follows: a commercial soybean protein preparation 60%,[5] and sucrose 40%, with the usual vitamin supplement.[6]

4. The determination of radioactivity in urine

Each urine sample was diluted to 200 ml; duplicate 50-ml aliquots were measured into Kjeldahl flasks, containing 1 mg of stable cobalt chloride as carrier and 2 ml of concentrated sulphuric acid. These samples were digested, with the addition of drops of concentrated nitric acid from time to time to aid the oxidation. After complete destruction of the organic matter, the contents of the flasks were transferred into beakers and the excess of acids evaporated off. The residue, consisting of inorganic salts, was transferred to planchets with water and the planchets dried. Radioactivity was counted with a Cyclotron Specialties Geiger-Muller counter with a window thickness of 3.1 mg/cm^2. To test the recovery of radioactivity, 2 or 4 mμg of the radioactive vitamin B_{12}, equivalent to 25 to 50 c.p.m. above background, respectively, were added to 50 ml of urine from normal rats receiving no radioactive substance, which was collected over a comparable period of two days. The recovery was approximately 75% of the calculated value. In similar experiments, higher recoveries were obtained when the amount of radioactivity added to the urine was greater. No effort was made to correct self-absorption due to the salt present in the urine.

[5] Sobee. Mead Johnson and Company.

[6] Vitamin supplement: For each 100 gm of ration are added vitamin A 1,260 I.U.; vitamin D 178 I.U.; α-tocopherol 2.5 mg; 2-methyl-1,4-naphthoquinone 0.2 mg; niacin 5.0 mg; inositol 10.0 mg; biotin 0.01 mg; *p*-aminobenzoic acid 25.0 mg; folic acid 0.02 mg; choline chloride 100.0 mg.

5. Experimental results

In the first experiment, 11 young deficient animals and 8 normal young male litter mates, kept in individual metabolism cages, were distributed between two subgroups (6 and 5 deficient, and 4 and 4 normal), of which one served as control. Each member of the second subgroup received subcutaneously 41.6 mμg of vitamin B_{12}* in two divided dosages for the first 5 days of the week and in one injection on Saturdays. No injection was given on Sundays. Thus, the total amount given to each rat per week was approximately 250 mμg. The precise amount administered is given in table 1, in c.p.m. (1 μg = 12.5 c.p.m.). All animals were offered the soybean protein-sucrose diet ad libitum and were weighed twice a week. Food consumption records were kept. Urinary collections were made at regular intervals.

Figure 1 shows the mean body weight changes of the 4 groups of animals. During the entire period of the experiment, injection of vitamin B_{12} markedly increased the growth rate of the deficient but not of the normal rats (compare curves 3 and 2). No difference was observed among the normal animals during the first three weeks of feeding; thereafter the B_{12}-dosed animals tended to grow faster. This therefore suggests the development of B_{12} deficiency during prolonged feeding of the soybean protein-sucrose diet. The stimulation in the growth rates of deficient and normal rats was accompanied by a marked increase in voluntary food consumption (Ling and Chow, '52). During 37 days, food intakes averaged 330 gm for the B_{12}-dosed and 300 gm for the control normal animals, 256 gm for the B_{12}-dosed and 144 gm for the control deficient animals.

Table 1 summarizes the data on the measurement of radioactivity in the urines of normal and deficient rats receiving the labelled vitamin B_{12}. During the 28 days of injection the amount of radioactivity found in the urine did not exceed 6 to 7% of that in the administered dose, regardless of the state of deficiency of the rats. Thus, no marked difference

in excretion patterns was observed. During periods J–K and L, when no B_{12}* was injected, the radioactivity in the urine was essentially the same as that found during the two preceding periods, H and I. This would indicate that a large portion of the administered vitamin B_{12}*, as measured by

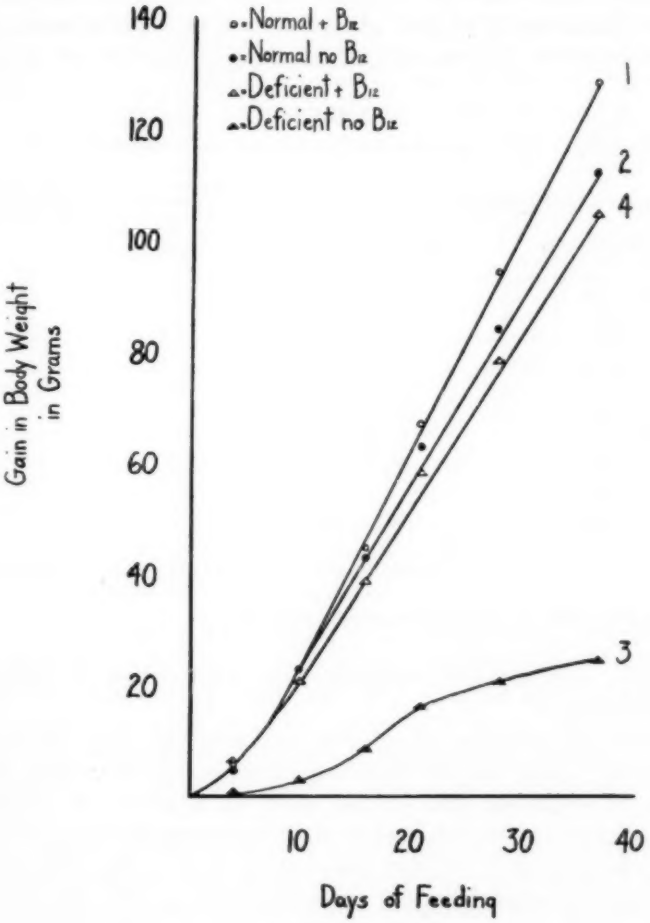

Figure 1

radioactivity, was not readily excreted. This observation is not at variance with the results of our earlier experiments (Chow, Barrows and Lang, '50), where essentially complete recovery in urine of injected vitamin B_{12} was achieved within 24 hours or less after a single parenteral injection of a massive dose (several hundred times that used here). It merely demonstrates that rat tissues can retain only a certain amount (1 to 2 μg per rat; Yamamoto et al., '51) of paren-

TABLE 1

Radioactivity in urine of normal and deficient rats injected with labelled vitamin B_{12}

PERIOD	NO. OF DAYS PER PERIOD	RADIOACTIVITY INJECTED PER PERIOD	RADIOACTIVITY IN URINE OF NORMAL RAT	RADIOACTIVITY IN URINE OF DEFICIENT RAT
		c.p.m.[1]	*c.p.m.*[1]	*c.p.m.*[1]
A	1	920	49	45
B	2	1,840	51	72
C	2	1,840	44	47
D	2	875	83	82
E	3	2,760	77	94
F	4	2,715	86	115
G	3	2,760	87	113
H	4	2,715	155	112
I	3	2,715	130	101
J	4	0	200	275
K	3	0	154	156
L	4	0	135	136
		Total 19,140	1,250	1,350

[1] Radioactivity is expressed in counts per minute.

terally administered vitamin B_{12}. Any excess is rapidly eliminated.

The low recovery of injected radioactivity from the urine of rats raised on the breeding stock or soybean stock diet can be explained also on the basis of retention of vitamin B_{12} by the tissues. That such retention occurs has been shown by Chow et al. ('51) and Rosenblum et al. ('52), who could account quantitatively for the unexcreted portion of injected vitamin B_{12} in the rat by measurements of radioactivity, and

by Harte and Chow ('52), who have reported on the storage of B_{12}* in tissues and organs.

Another possible, though unlikely, explanation for the low recovery might be the faulty collection and measurement of radioactivity. To rule out this possibility we injected a group of deficient young rats with $Co^{60}Cl_2$. The salt was administered subcutaneously in doses adjusted to provide approximately the same amount of radioactivity as was administered in the preceding experiment. Twelve rats (9 males and three females) from three litters, ranging in age from 35 to 50

TABLE 2

Radioactivity of urine of deficient rats injected with radioactive $Co^{60}Cl_2$

PERIODS	DAYS PER PERIOD	RADIOACTIVITY INJECTED	RADIOACTIVITY RECOVERED IN URINE
		c.p.m.[1]	*c.p.m.*[1]
I	1	1,305	730
II	2	1,305	560
III	4	2,610	1,130
IV	3	2,610	2,080
V	4	2,610	1,520
VI	3	2,610	1,310
VII	4	1,305	900
VIII	2	0	400
	Total	14,355	8,630

[1] Radioactivity is expressed in counts per minute.

days, were used. Six (4 males and two females) received the radioactive cobalt and 6 (5 males and one female) were used for controls. Weight and food consumption records were kept and urine and stool specimens were collected at intervals during 21 days. These injections had no effect on the growth rate or food intake of rats raised on the soybean stock diet.

The results of assays for radiosensitivity in urine are shown in table 2. In contrast to the vitamin B_{12} experiment, here a major fraction, 60%, of the injected radioactivity appeared in the urine. The excretion of radioactivity decreased mark-

edly, but not completely, and promptly after dosage was stopped.

The remaining portion of the administered dose undoubtedly was retained in the tissues (Comar and Davis, '47) or excreted in the fecal matter. As a matter of fact, determination of total radioactivity in the stool specimens collected during the entire experimental period demonstrated the presence of as much as 15% of the injected vitamin.

These findings do not indicate that the low recoveries of radioactivity in the urines of animals receiving radioactive B_{12} reflect faulty techniques of collection or estimation of radioactivity, but rather suggest that in the dosage range studied, a significant fraction of the amount of vitamin B_{12} injected is retained in the tissues.

The second experiment was designed to compare the excretion of orally and parenterally administered vitamin B_{12} by deficient and saturated adult animals. To this end, 6 deficient rats and an equal number of saturated rats were placed in individual metabolism cages. Three animals from each group were injected subcutaneously with a single dose of 500 µg of vitamin B_{12}* and the other three received the same amount by mouth. Urine specimens were collected during the day immediately preceding administration and daily thereafter for 4 consecutive days. All urine samples were analyzed for radioactivity and for vitamin B_{12} by microbiological assay.

Under these experimental conditions, the fraction of the parenterally administered radioactive vitamin which appeared in the urine, as measured by excreted radioactivity, was only slightly lower in the deficient animals than in the saturated animals. Values of 8.9, 6.9 and 7.3% of the counts injected were found in the urines of the saturated animals; 5.9, 5.2 and 5.4% in the deficient animals. With both types of animals, the greatest output was seen in the first collection period, 24 hours after the injection of vitamin B_{12}*. The output decreased sharply in all the animals thereafter.

Analyses for total microbiological activity demonstrated that basal urine specimens of the saturated rats contained from two to three times as much vitamin as those of the depleted animals. In the saturated animals the urines showed the highest microbiological activities in the second collection period. In the deficient animals the differences in microbio-

TABLE 3

The appearance of microbiological activity and radioactivity in urine of rats following oral and subcutaneous administration of labelled vitamin B_{12}

TYPE OF ANIMAL:	SATURATED RATS		DEFICIENT RATS	
Time from administration (hours)	MB [1]	R [2]	MB [1]	R [2]
	mμg	*c.p.m.*	*mμg*	*c.p.m.*
	Subcutaneous administration			
−24	179 ± 31	0	75 ± 2	0
+24	302 ± 50	227 ± 25	89 ± 16	155 ± 10
+48	378 ± 131	64 ± 2	75 ± 27	66 ± 12
+72	171 ± 28	56 ± 7	59 ± 13	31 ± 3
+96	176 ± 30	54 ± 4	71 ± 17	24 ± 9
	Oral administration			
−24	143 ± 32	0	70 ± 6	0
+24	220 ± 20	4 ± 8	60 ± 19	4 ± 8
+48	231 ± 20	31 ± 5	47 ± 8	48 ± 21
+72	129 ± 13	33 ± 14	56 ± 10	16 ± 4
+96	127 ± 7	19 ± 6	40 ± 7	20 ± 8

[1] MB = microbiological activity of vitamin B_{12} in millimicrograms.
[2] R = radioactivity of Co^{60} in counts per minute.

logical activity in the urines from different collection periods were not marked.

After oral administration of vitamin B_{12}*, only low levels of radioactivity were found in the urine samples. The differences in excreted radioactivity between the deficient animals and the saturated animals were negligible. Unlike similar groups of rats which received B_{12} subcutaneously, those given the vitamin orally showed, without exception, the greatest output of radioactive material in their urines after the first 24-hour collection period.

In the next experiment deficient, normal and saturated animals (6 in each group) were divided into two subgroups to which 500 μg of vitamin B_{12}* or an amount, 20 mμg, of $Co^{60}Cl_2$ of equivalent radioactivity, were given subcutaneously in one single dose. Urine specimens were collected as usual at desired intervals and analyzed for radioactivity and for vitamin B_{12} by microbiological assay. On the 11th day, those animals which had received vitamin B_{12}* previously were injected subcutaneously with three 50-μg doses of vitamin B_{12} at 10:00 A.M., 1:00 P.M. and 4:00 P.M. Urine samples were collected and tested for radioactivity.

TABLE 4

Variations in the microbiological activity in basal urine

DAYS BEFORE INJECTION	TYPE OF RATS		
	Saturated	Normal	Deficient
	mμg	*mμg*	*mμg*
1	406 ± 33	62 ± 2	52 ± 6
2	243 ± 16	67 ± 4	62 ± 2
3	290 ± 17	86 ± 6	81 ± 6
4	130 ± 9	53 ± 4	56 ± 4

The microbiological activity in the basal urine specimens (table 4) was consistently higher in the saturated animals than in the normal or deficient animals. The difference between the averages of the latter two groups was not statistically significant.

Urinary excretion of administered vitamin B_{12}* by saturated, normal and deficient animals is shown in table 5. The data demonstrate that the radioactivity in the urine specimens of the three groups of animals receiving vitamin B_{12}* amounted to 2 to 3% of the administered dose. No significant differences were observed among the three types of rats. Undoubtedly, all of the animals were able to retain essentially all of the administered vitamin B_{12}*. It is possible that if a larger dose were administered, differences might appear. Administration of $Co^{60}Cl_2$ was followed by

the appearance of a much larger fraction of the injected salt in the urines of the three groups of animals. The microbiological activity in the urines of the saturated animals was found again to be much higher than in the urines of the normal and deficient rats.

TABLE 5

The appearance of microbiological activity and radioactivity in urine of rats following subcutaneous injection of labelled vitamin B_{12} or of $Co^{60}Cl_2$

HOURS AFTER ADMINISTRATION	SATURATED		NORMAL		DEFICIENT	
	Mb [1]	R [2]	Mb [1]	R [2]	Mb [1]	R [2]
	mμg	*c.p.m.*	*mμg*	*c.p.m.*	*mμg*	*c.p.m.*
			Vitamin B_{12}			
+ 24	252 ± 13	97 ± 5	77 ± 10	98 ± 12	52 ± 5	83 ± 4
48	259 ± 9	19 ± 9	78 ± 5	31 ± 5	60 ± 9	15 ± 10
Total recovery (%)	2.3 ± 0.3		2.6 ± 0.3		2.0 ± 0.3	
			$Co^{60}Cl_2$			
24	232 ± 12	1,470 ± 176	70 ± 11	955 ± 116	62 ± 10	1,290 ± 216
48	266 ± 9	101 ± 18	69 ± 15	81 ± 26	48 ± 2	84 ± 32
Total recovery (%)	78 ± 9		52 ± 5		69 ± 10	

[1] MB = microbiological activity of vitamin B_{12} in millimicrograms.
[2] R = radioactivity of Co^{60} in counts per minute.

DISCUSSION

Parenteral administration of radioactive vitamin B_{12}, at a dosage of approximately $\frac{1}{2}$ μg per rat, to normal, deficient or saturated animals, either weanling or adults, resulted in the appearance of radioactivity in the urine to the extent of less than 10% of the administered dose. The remaining portion was retained in the organs and tissues. Although the data suggest that the deficient adult animals retained more vitamin B_{12} than the normal ones, the number of animals used and the accuracy of the determinations do not justify such a conclusion at this time. No such difference was observed with the young rats.

Urinary excretion after injection of a vitamin has been used as an index of the extent of body reserve of the vitamin. Thus, the so called saturation test (Klein and Eheart, '44) has become one of the principal means of determining adequacy of vitamin C intake. In practice, it was found that after adequate dietary intake of ascorbic acid, the test subjects excreted 50% or more of the administered dose in the urine 24 hours after injection. Failure to excrete that percentage of the test dose indicated a need for an increase in daily intake.

Under these experimental conditions, the absence of significant differences in the urinary excretion patterns of rats with different degrees of vitamin B_{12} reserve does not necessarily mean that such differences do not exist. As a matter of fact, comparisons of the microbiological activity in the basal samples support the idea that the degree of B_{12} sufficiency is reflected in the urinary excretion. However, interpretation of the excretion data is further complicated by the fact that the administration of vitamin B_{12}, by injection or by mouth, results in retention of the injected vitamin in certain organs, such as kidney, liver, pancreas, and so forth, where it is not released over a period of weeks, if not months (Harte and Chow, '52). The tenacity with which this vitamin is held by these tissues is suggested by the experiment herein reported, in that the injection of stable vitamin B_{12} in large quantities did not in any way release the vitamin B_{12}^* previously administered to these animals. If the vitamin B_{12}^* retained by these tissues could be released readily, one would expect to find radioactivity in the urine following the injection of vitamin B_{12}. Actually, virtually no significant increase in radioactivity in the post-injection urine specimens was found.

SUMMARY

Vitamin B_{12} labelled with Co^{60} was injected subcutaneously in small dosages in normal as well as deficient weanling rats daily, except Sundays, for approximately 4 weeks. The

growth rates of B_{12}-dosed and of control animals of different degrees of B_{12} reserves were compared. Food consumption records were also kept. The measurement of radioactivity in the urines of both normal and deficient rats demonstrated no significant differences in excretion patterns, amounting to only a few per cent of the administered vitamin. The recovery of radioactivity in urine was much greater when $Co^{60}Cl_2$ was injected in deficient rats.

Similar excretion experiments were performed with adult rats. An additional group of rats previously "saturated" with stable vitamin B_{12}, both by injection and by oral feeding, was included. It was found that the basal urinary excretion was several-fold greater than that of either the normal or deficient rats. However, even then, no marked difference in excretion pattern among the three groups of rats was observed.

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THE BIOLOGICAL ACTIVITY OF DIETHYL RIBOFLAVIN [1, 2]

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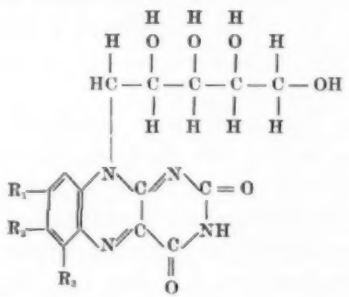
SEVEN FIGURES

(Received for publication March 11, 1952)

Of the 7 analogs of riboflavin I which contain a ribityl chain, three are able to replace riboflavin in the nutrition of rats and the bacteria *Lactobacillus casei* and *Bacillus lactis acidi*. These three are 6-methyl-9-(D-1′-ribityl)-isoalloxazine II, 7-methyl-9-(D-1′-ribityl)-isoalloxazine III, and 6-ethyl-7-methyl-9-(D-1′-ribityl)-isoalloxazine IV.



	R_1	R_2	R_3
I	CH_3-	CH_3-	H-
II	H-	CH_3-	H-
III	CH_3-	H-	H-
IV	CH_3-	C_2H_5-	H-

	R_1	R_2	R_3
V	H-	CH_3-	CH_3-
VI	Cl-	Cl-	H-
VII	C_2H_5-	H-	H-
VIII	C_2H_5-	C_2H_5	H-
IX	-C_2H_4-	-C_2H_4-	H-

[1] This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

[2] This investigation was supported in part by a grant-in-aid from the Fluid Research Fund Committee of the School of Medicine and Dentistry, University of Rochester.

While Karrer and Strong ('35) claimed activity, and Kuhn et al. ('37) claimed inactivity for the 6-methyl analog at a level of 10 μg per day, both groups of workers have shown it to be about 30% as active as riboflavin itself in rats when 20 μg per day are given. Karrer et al. ('35) have presented evidence that the 7-methyl analog has about 30% activity in rats at levels of either 10 or 20 μg per day, but merely state (Karrer and Quibell, '36) that at 10 μg per day, the 6-ethyl-7-methyl is very active.

When tested for riboflavin activity on *Lactobacillus casei*, the 6-methyl, 7-methyl and 6-ethyl-7-methyl analogs showed about 41, 65 and 90%, and on *Bacillus lactis acidi* about 43, 81 and 71% of the activity of equivalent amounts of riboflavin, respectively (Snell and Strong, '39).

Two of the 7 analogs have been shown to be riboflavin inhibitors. The analog 5,6-dimethyl-9-(D-1′-ribityl)-isoalloxazine V (isoriboflavin) has displayed this property in rats (Emerson and Tishler, '44), while 6,7-dichloro-9-(D-1′-ribityl)-isoalloxazine VI (dichlororiboflavin) has been shown to be an inhibitor for *Staphylococcus aureus* and *Streptobacterium plantarum* (Kuhn et al., '43).

It appears that only those riboflavin analogs which are able to serve as the sole flavin in the nutrition of *L. casei* and *B. lactis acidi* are also able to perform this function in the rat. The finding that the 7-methyl and the 6-ethyl-7-methyl analogs were capable of these functions, while 7-ethyl-9-(D-1′-ribityl)-isoalloxazine VII was not (Karrer, '36), made it of interest to synthesize 6,7-diethyl-9-(D-1′-ribityl)-isoalloxazine VIII (diethyl riboflavin, Lambooy, '50) to investigate its activity for bacteria which require riboflavin, and for rats.

It was found that at limiting concentrations, diethyl riboflavin has 90%, and at greater than limiting concentrations, 80% of the activity of riboflavin for *L. casei* 7469 (Lambooy, '51). We are now able to report that diethyl riboflavin also posseses from 80 to 90% riboflavin activity for *B. lactis acidi* at limiting concentrations.

In view of all previous findings concerning the ability of riboflavin analogs to function as riboflavin, one might conclude, on the basis of the bacterial growth studies, that diethyl riboflavin should be as good as, if not superior, to the 6-ethyl-7-methyl analog in the nutrition of the rat. While diethyl riboflavin as the only flavin in the diet of rats results in weight maintenance or small gains during periods of weeks at low levels (3.23 μg to 64.6 μg/day), and very large gains during periods of days at intermediate levels (8.08 μg to 32.3 μg/gm of food), this cannot be considered to be due to true riboflavin-like activity, because the animals fail to survive. It will be seen that at still higher levels (2 mg/day) not only is diethyl riboflavin devoid of riboflavin activity for the rat, but it is by far the most potent inhibitor of this activity among the analogs of riboflavin yet described.

The lowest levels tested showed no growth activity, but since the experimental animals did maintain their weights the level was elevated again and again. When the animals consumed 16.2 μg (equivalent to 15 μg of riboflavin) of diethyl riboflavin per gram of food, they showed astonishing weight gains, often exceeding the rate of gain of animals receiving 15 μg of riboflavin per day. When the level was elevated to 32.3 μg per gram of food, the animals failed to show these gains consistently, and it appeared that an aspect of inhibition was becoming evident. In all, 11 levels of the diethyl analog were administered. To study the inhibitor properties of diethyl riboflavin, 12 groups of animals were fed high levels of the analog with or without additional riboflavin.

As far as the nutrition of the rat is concerned it appears that the data are best explained on the basis of a dual inhibitor role for diethyl riboflavin. First, it appears that low and intermediate levels of the analog are capable of mobilizing riboflavin from the tissue stores of deficient animals by a displacement mechanism. Second, at high levels of intake real inhibition becomes predominant, but this inhibition is not devoid of a contribution similar to that described for the first role.

METHODS

To conserve space the following notations will be used. In graphs and tables, riboflavin and diethyl riboflavin will be referred to as R and D, respectively. The low and intermediate levels of diethyl riboflavin were equimolar with corresponding levels of riboflavin. Since the molecular weight of diethyl riboflavin is 404 and that of riboflavin 376, equimolar solutions would contain 3.23 μg and 3.00 μg, respectively, and to indicate this the levels of diethyl analog will be designated as, for example, 3 μg equivalents (3 μg equivalents of diethyl riboflavin) and multiples of this.

Since this is the report of three distinct studies, it will be advantageous to describe the particulars of the methods used and results obtained under separate headings: (1) Bacterial Growth; (2) Rat Growth; and (3) Rat Growth Inhibition.

(1) Bacterial growth

The organism used was *Bacillus lactis acidi* which was obtained from Dr. Elizabeth McCoy, Department of Agricultural Bacteriology, University of Wisconsin. With the exception of the organism, the study was largely a repetition of that performed earlier with *Lactobacillus casei* (Lambooy, '51; see fig. 1). One additional growth pattern was studied for both *B. lactis acidi* and *L. casei;* this involved the measurement of acid production at various time intervals throughout the 120-hour growth period. Equimolar quantities of flavin equivalent to 8.22×10^{-10} moles per tube were used (fig. 2).

(2) Rat growth

A colony of 30 weanling Wistar rats was divided into 10 groups of three animals each consisting of two females and one male. These animals were freed of vermin and kept in individual cages in a temperature-controlled room. Clock-operated lights gave 12 hours of light each day. All ani-

mals were placed on a riboflavin-deficient diet and fed ad libitum until growth had reached a plateau to the extent that an animal failed to gain 6 gm in a period of 14 days. The diet consisted of [3]: sucrose 680 gm, vitamin-free casein 180 gm, hydrogenated vegetable oil 100 gm and U.S.P. salt mixture No. 2, 40 gm. To each kilogram of this diet were added 20 gm cod liver oil, 50 mg each of vitamin K_1, nicotinamide and *p*-aminobenzoic acid, 10 mg each of thiamine chloride hydrochloride and pyridoxine hydrochloride, 25 mg calcium pantothenate, 100 mg *i*-inositol, 1 gm choline chloride, 0.6 mg each of biotin and pteroylglutamic acid and 30 μg of vitamin B_{12}. Each rat received 5 mg of mixed tocopherols in corn oil once each week.

When deficient, 5 groups of three animals each were placed on the following levels of riboflavin: 3, 6, 9, 12 and 15 μg per day. A corresponding set of 5 groups of three animals each were placed on equivalent levels of diethyl riboflavin per day. These supplements were administered by pipette in the form of a water solution containing 100 μg and 107.6 μg per milliliter, respectively. Once the animals were placed on the flavin supplements, ad libitum feeding was continued and the daily food consumptions were recorded throughout the entire experiment. This plan of supplementation was continued for a period of 28 days. Body weight changes were plotted as per cent of the starting weight gained or lost during the 28 days for each animal and then the mean curve for a group constructed (fig. 3).

With the exception of two animals, those which had received 12 and 15 μg riboflavin were sacrificed at the end of the 28 days; all others were fed various levels of diethyl riboflavin. Throughout the rest of this growth study the remainder of the animals were selected on one of the following bases for modification of the level of supplementa-

[3] The authors are indebted to Merck and Co., Inc., Rahway, New Jersey, for generous gifts of crystalline vitamin B_{12} and vitamin K_1 used in these studies.

tion: either the animal had failed to gain 6 gm during the last 14 days of supplementation or it was brought to this condition by being deprived of riboflavin. As a result of these procedures, it was possible to use some animals for study of a total of 5 supplement levels. Animals of the R-3, R-9, and one animal of the R-15 groups, were changed from a flavin supplement of 3, 9 and 15 μg R per day to 3 μg R + 15 μg equivalents D, 9 μg R + 9 μg equivalents D and 15 μg R + 15 μg equivalents D per day, respectively, for a period of 21 days to discover if diethyl riboflavin would have growth-stimulating properties in the presence of riboflavin.

When it appeared that a minimum of 15 μg equivalents of diethyl riboflavin might be required for growth, the supplements were increased so that eventually 30 μg equivalents and 60 μg equivalents D were given each day (fig. 4). Higher levels than this are not conveniently given by pipette, so the analog was mixed in the diet at levels of 7.5, 15 and 30 μg equivalents of diethyl riboflavin per gram of food (fig. 5).

(3) Rat growth inhibition

Weanling female Wistar rats were caged under the conditions described above and fed for 4 to 6 days the diet already described except that it contained a somewhat higher level of *p*-aminobenzoic acid. The animals were then reassembled so that groups which were to be directly compared had the same average weight. For the 12 groups used, this average weight was 42 gm. These groups then received, just prior to access to their food, the particular flavin supplements assigned to them. The flavin supplements as listed in table 2 were administered by stomach tube in 0.5 ml of a 6% gum acacia suspension. The level of riboflavin which was administered with the analog was increased until a level was reached which produced approximately a 50% reversal of the inhibition (fig. 6).

RESULTS

(1) Bacterial growth

Figure 1 shows the growth of the organism on diethyl riboflavin as measured by acid production. At the end of 120 hours, the growth on the diethyl analog was equivalent to 88% of that on riboflavin. From figure 2 it can be seen that acid production during the first 24 hours is somewhat more rapid on riboflavin but that after this early period the rate of acid production is essentially the same. At the end of 120 hours the activity of the diethyl riboflavin was equivalent to 90% of that of riboflavin for *B. lactis acidi* and about 80% for *L. casei* 7469.

(2) Rat growth

The pertinent findings are summarized in table 1 and figures 3, 4 and 5. In spite of the limited number of animals used, the grams of weight gained per day for the 3-R and 6-R groups are independently significantly different from those of all other riboflavin groups (all p values $= < 0.001$). As might be expected from consideration of figure 3, the higher levels (9-, 12- and 15-R) do not differ among themselves. The variations in grams of food consumed per gram gained per 100 gm body weight were too great, causing the variance (F) to be too large for the results to be properly compared by statistical methods.

The effect of adding diethyl riboflavin to small amounts of riboflavin were studied. Animals receiving 3 μg + 15 μg equivalents D, 9 μg R + 9 μg equivalents D and 15 μg R + 15 μg euivalents D grew at a rate essentially consistent with the level of riboflavin administered.

The suggestion that 15 μg equivalents of diethyl riboflavin did stimulate growth led us to try this level again and also levels of 30 μg equivalents and 60 μg equivalents. Each time the level of diethyl riboflavin was doubled the weight gained per day was doubled. The intermediate levels of

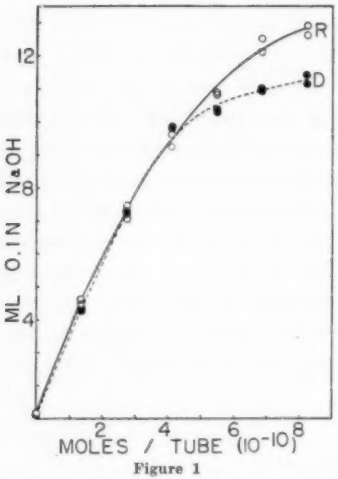


Figure 1

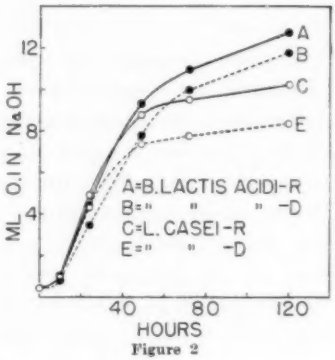


Figure 2

Fig. 1 Acid production by *Bacillus lactis acidi* in the presence of riboflavin (R) and diethyl riboflavin (D). One hundred and twenty-hour growth period.

Fig. 2 Acid production by *Bacillus lactis acidi* and *Lactobacillus casei* at various time intervals during a 120-hour growth period in the presence of riboflavin (R) and diethyl riboflavin (D).

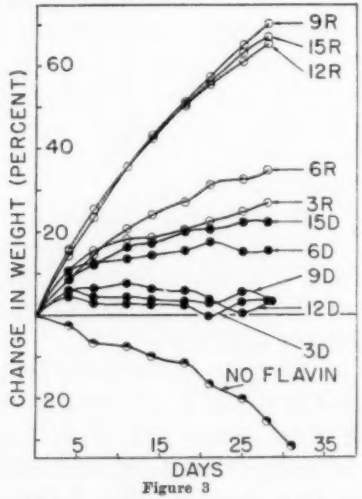


Figure 3

Fig. 3 Change in weight of rats on various low levels of riboflavin (R) and diethyl riboflavin (D). Oral administration.

the analog could not be conveniently administered by pipette, so the analog was mixed in the diet at three levels. The findings are best described under separate subheadings.

Seven and one-half micrograms e D per gram food. Five animals were used; they were fed this diet until they died. The average survival time on this regime was 32 days, with one animal surviving 51 days. They gained at the aver-

TABLE 1

Weight gains in grams per day of rats on various low levels of riboflavin (E) and equivalent amounts of diethyl riboflavin (D)

SUPPLEMENT	NO. OF ANIMALS	DAYS	GM GAINED PER DAY	AVE. WT. GAINED, GM
3-R/day	3	28	0.89	25.0
6-R/day	3	28	1.30	36.3
9-R/day	3	28	1.92	53.7
12-R/day	3	28	2.21	62.0
15-R/day	3	28	2.38	66.7
3-D/day	3	28	0.09	2.5
6-D/day	3	28	0.45	12.7
9-D/day	3	28	0.07	2.0
12-D/day	2	28	0.13	3.5
15-D/day	3	28	0.92	25.7
15-D/day	3	21	0.26	
30-D/day	3	21	0.57	
60-D/day	3	21	1.00	
7.5-D/gm food	See text for details.			
15-D/gm food	See text for details.			
30-D/gm food	See text for details.			

age rate of 2.0 gm per day during the first 11 days, the maximum being 4.0 gm per day for this period. The average weight then declined at the rate of 1.3 gm per day for the next 10 days. The animals then gained again at an average rate of 4.0 gm per day for 4 days, during which time one animal gained at the rate of 6.8 gm per day. After the 25th day the average weight again began to fall and the animals began to die.

Fifteen micrograms e D per gram food. Eleven animals which had received no riboflavin for periods of time ranging from 78 to 92 days were used. They had, however, received varying low levels of the diethyl analog for periods ranging from 33 to 49 days during this time. This group was fed the diet until they died, the average survival time being 28 days, one animal surviving for 41 days. These animals gained at the average rate of 2.8 gm per day for 7 days, the maximum rate being 4.3 gm per day. The average weight then declined at the rate of 0.8 gm per day for the next 7 days. The animals then gained weight for the next 14 days at the average rate of 1.4 gm per day, the maximum rate being 4.0 gm per day. The greatest growth rate shown by any of these animals was 5.8 gm per day for 4 days. It was characteristic of these animals to die unexpectedly. Their otherwise normal behavior is indicated by the fact that the average amount of food consumed on the day of death was 8.7 gm, the maximum being 10.1 gm.

Thirty micrograms e D per gram food. Two animals were used; they were fed the diet for 35 and 48 days, respectively, these being the survival times on this regimen. These animals gained at the average rate of 2.0 gm per day for 7 days, the maximum being 3.4 gm per day. They lost weight for the next three days at the average rate of 5.0 gm per day. The next 4 days showed an average gain of 4.1 gm per day, the maximum being 5.8 gm per day. This cycle of weight changes was repeated twice more at intervals of from 4 to 10 days but not concomitantly, and the drift of the average weight began to be downward until death.

At no time could a change be observed in the daily food consumed following administration of the analog by pipette. This was true even at a level of 120 μg equivalents D per day, which was tried for 5 days but abandoned because of its inconvenience. However, in all cases, when the analog was mixed with the food the amount consumed increased enormously, even the first day the new diet was offered (fig. 7). These increases were reflected in the large weight gains

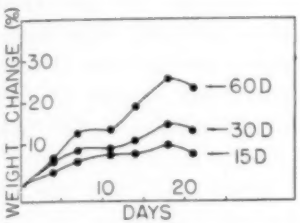


Figure 4

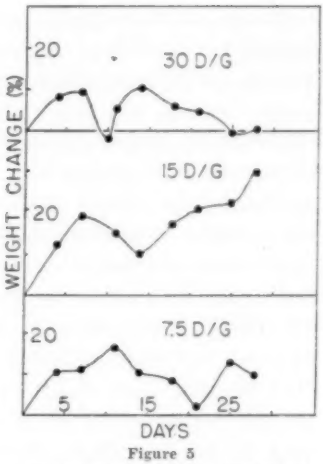


Figure 5

Fig. 4 Change in weight of rats on 15 μg equivalents to 60 μg equivalents of diethyl riboflavin per day. Oral administration.

Fig. 5 Change in weight of rats on 7.5 μg equivalents, 15 μg equivalents and 30 μg equivalents per gram of food.

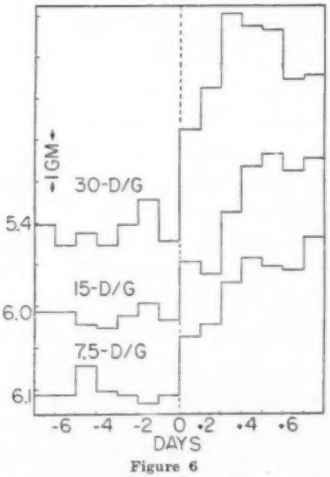


Figure 6

Fig. 6 Increased food consumption following the incorporation of diethyl riboflavin in the diet.

and represented approximately a 50% increase in food consumed by the 7.5 μg equivalents D per gram group by the 4th day; a 65% increase for the 15 μg equivalents D per gram group by the 4th day; and a 100% increase for the 30 μg equivalents D per gram group by the third day. The magnitude of this change can be illustrated by the case of one animal in the 7.5 μg equivalents D per gram group. During the 10 days preceding the addition of the analog to the diet, the animal consumed an average of 6.6 gm per day, not varying from this value by more than 1 gm. The 10th day on the 7.5 μg equivalents D diet the animal consumed 14.0 gm of food, and had consumed an average of 11.5 gm per day for this period. The daily average food consumption for this animal for a period of 28 days on 3 μg of riboflavin per day was 6.5 gm per day! Since all three groups showed cycles of weight gain and loss, it is obvious that they ate almost nothing during periods of loss; frequently at these times the animal appeared to be moribund and a few died. Most of them resumed eating and showed again very large weight gains.

Observations concerning survival time require brief comment. One animal was brought into a riboflavin-deficient state in 53 days. It was then kept on a level of 15 μg equivalents of diethyl riboflavin per day for a period of 109 days before it was sacrificed. Even though this animal had lived for 162 days without riboflavin, it showed a net gain of 25 gm during the 109 supplemented days. When sacrificed, this animal appeared essentially normal.

Of those animals used in the original study of growth at various levels of riboflavin, only those which had received 3, 6 or 9 μg of riboflavin were used for the study of various levels of diethyl riboflavin. With no regard to the level of analog used, the average survival time on the analog was 52 days. The average survival time of these animals from the beginning of the depletion period was 125 days, during which time some had received as much as 9 μg riboflavin for a period of 49 days. On the other hand, those animals which had received no riboflavin from the beginning of the original

depletion period had an average survival time of 119 days. Thus, although the first group had received limited amounts of riboflavin for from 38 to 49 days, the average gain in their total survival time was only 6 days.

Early in the study we questioned whether the animals were absorbing the analog. Quantitative measurements were made of the weekly fecal excretion of total flavin by the use of *L. casei* 7469. Groups showed the following average levels of material which were measured as "riboflavin activity"; flavin-free, 39 μg/week; 15 μg equivalents D per day, 26 μg per week; 30 μg equivalents D per day, 31 μg per week; and 60 μg equivalents D per day, 28 μg per week.

(3) Inhibition studies

The results are summarized in table 2 and figure 6. Animals fed a flavin-deficient diet gained 9 gm and those fed 2 mg of diethyl riboflavin gained 10 gm during the 21 days of the test. The final average weights of these two groups was not significantly different ($p = 0.545$). While all of the animals on the flavin-deficient diet survived, only 38% of those receiving the diethyl riboflavin lived for the duration of the test period. By the 5th day of the respective regimens, the "deficient" group had gained an average of 4 gm, while those receiving 2 mg of the diethyl analog gained an average of 8 gm. This difference is highly significant ($p = < 0.001$).

Survival of animals on 2 mg diethyl riboflavin did not reach 100% until the supplement also provided 80 μg of riboflavin per day. This level accomplished only about 30% reversal of the inhibition caused by the analog, as expressed in terms of weight gain. Not until 300 μg of riboflavin were administered simultaneously with the 2 mg of diethyl riboflavin did the reversal amount to approximately 50% as compared to the growth shown by the group receiving 300 μg of riboflavin alone. It was felt that little value could be gained by increasing the level of riboflavin until complete reversal was accomplished.

TABLE 2

Ultimate weights and per cent survival of groups of rats on flavin-deficient diets supplemented with 2 mg diethyl riboflavin (D) and various levels of riboflavin (R), for 21 days

GROUP NO.	SUPPLEMENT	NO. OF ANIMALS	NO. OF ANIMALS SURVIVING	PER CENT SURVIVING	WEIGHT GAINED, GM	PROBABILITY (P)
1	Flavin-deficient	11	11	100	9	
						0.545
2	2 mg D/day	13	5	38	10	
3	9 μg R/day	6	6	100	38	
						0.001
4	9 μg R + 2 mg D/day	9	6	67	10	
5	40 μg R/day	6	6	100	52	
						0.001
6	40 μg R + 2 mg D/day	21	12	57	13	
7	80 μg R/day	7	7	100	64	
						0.001
8	80 μg R + 2 mg D/day	11	11	100	18	
9	150 μg R/day	7	7	100	66	
						0.001
10	150 μg R + 2 mg D/day	11	11	100	20	
11	300 μg R/day	7	7	100	69	
						0.001
12	300 μg R + 2 mg D/day	13	13	100	38	

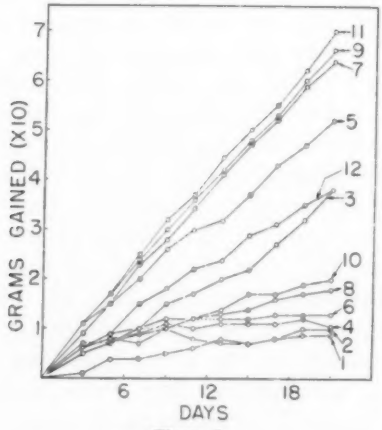


Figure 7

Fig. 7 The inhibition caused by diethyl riboflavin and the reversal of this inhibition by riboflavin. (See table 2 for key to groups.)

In all cases the growth of animals receiving 2 mg of diethyl riboflavin plus varying amounts of riboflavin (groups 4, 6, 8, 10 and 12) was significantly less than that of animals receiving the same amount of riboflavin alone (groups 3, 5, 7, 9 and 11). The differences between related pairs was highly significant (all p values $=<0.001$).

DISCUSSION

One cannot deny that rats which are deficient in riboflavin can be made to grow when they are given diethyl riboflavin. Further, the ocular lesions which frequently appear as a result of riboflavin deficiency and which appeared in some animals in varying degrees, are greatly improved within 24 hours after the administration of intermediate levels of the diethyl analog. If these effects were due to the ability of the diethyl riboflavin molecule to function in place of the riboflavin molecule in the biochemical processes involved, the animals would not have died. Since the animals did die, we must conclude that diethyl riboflavin cannot be utilized in the place of riboflavin in the nutrition of the rat.

This forces us to the conclusion that when the analog is administered it makes some riboflavin available to the animal. This riboflavin cannot arise by conversion of the diethyl riboflavin molecule or the animals would have lived. It cannot arise as a result of diethyl riboflavin's stimulating intestinal bacteria to synthesize riboflavin which was absorbed by the rat. Such synthesis would be accomplished by bacteria which would require no riboflavin and are, therefore, not dependent on diethyl riboflavin, or by organisms which require riboflavin. Our bacterial studies indicate that two of the latter group do not do this. No evidence obtained to date indicates that organisms can convert an ethyl group into a methyl group. The rats on a flavin-free diet excreted more "riboflavin activity" in the feces than did those animals receiving 60 μg equivalents of diethyl riboflavin per day.

This means that if the response is due to riboflavin, it must come from that stored in the tissues of the deficient ani-

mal. It is known that riboflavin deprivation for periods of several weeks does not exhaust the tissue stores of the vitamin. In fact, surprisingly large amounts are still to be found in the tissues of animals which have died as a result of such deprivation. This riboflavin is, therefore, unavailable to these animals by virtue of the fact that it must be so tightly bound that it cannot be readily released. It must be, for the most part, inactive biochemically, for if it were not, the animal could accomplish a higher degree of depletion.

That diethyl riboflavin is both chemically and physically very similar to riboflavin is clear from its chemical and physical properties and from its ability to replace riboflavin in the nutrition of at least two strains of bacteria which require riboflavin. This means that diethyl riboflavin can serve as the basic unit in the formation of the necessary coenzymes required by these bacteria.

It is equally evident that in the rat, diethyl riboflavin-containing coenzymes, if they are formed, cannot serve as coenzymes for at least one and possibly more apoenzymes. Yet, because of its physical and chemical similarity to riboflavin, it seems plausible that diethyl riboflavin might go into the tissue and there be exchanged for the stored riboflavin, which could then be made available for metabolic requirements.

If this were the case, then one might expect the following types of response: (a) At low levels of intake, diethyl riboflavin would make available equimolar amounts of riboflavin. This could never be more than an amount equivalent to the daily ingestion of the analog and would probably be less, due to the rapidity of excretion. It could, conceivably, make it possible for an animal to maintain its weight and to a large extent, maintain its appearance. This fits our experimental observations. (b) Somewhat higher levels would at first cause the release of sufficient riboflavin to permit some real weight gain. This gain would soon reach a plateau because at a constant level of administration the blood concentration would never be higher than a certain level. All of the riboflavin available through displacement at this level of concentra-

tion would soon be exhausted. Continued maintenance at this level would eventually cause the animal to show deficiency signs of varying degrees of intensity. Our growth curves on 30 and 60 μg equivalents per day show this characteristic. Since many of these animals had been kept on low levels of the analog for relatively long periods of time, about half of them finally reached a point where they showed mild characteristic signs of riboflavin deficiency, slight dermatitis, ocular exudate and unkempt coats. (c) Still higher levels such as those attainable by mixing the analog in the diet would lead to death in a relatively short time. The large ingestion of the analog would in effect wash the riboflavin out of the tissues in waves. The resulting sense of well-being would lead the animal to greater and greater food consumption and thus bring about a continued flow of displaced riboflavin. If for some reason such an animal should fail to consume its diet for a day, it would rapidly develop a biochemically functional deficiency of riboflavin. This might cause death, as it did in some of our animals, or near death at the peak of weight gain, or a precipitous loss in weight due to failure to eat. If the animal survived, hunger would once more drive him to eat and thus start the cycle again. Eventually a point would be reached where no more riboflavin would be available and the animal would promptly die. This is essentially the pattern shown by the animals which received the analog in the diet. Furthermore, once on these regimens, these animals promptly recovered from any external signs of riboflavin deficiency which they may have displayed. When they died, they seemed to be essentially normal in external appearances.

There remain two more points which require interpretation. The first is the failure of the animals which received 60 μg equivalents of diethyl riboflavin (and the few which received 120 μg equivalents for 5 days) to show more rapid gains in weight. The flavin administered at these levels was completely dissolved in water. It was found that 0.1 ml of solution given by pipette at one time was all a rat could

handle for some minutes. Thus, it was customary to administer the solution in 0.1-ml lots every 10 minutes until consumed. For 60 μg equivalents and 120 μg equivalents, one and two hours, respectively, were required. Since no food was available until after the supplement had been given, the concentration of the flavin never reached a high level in the blood, either because it was excreted or because it liberated riboflavin which was excreted. The ingestion of the flavin with the food would permit the concentration of the flavin to go up rapidly, and such riboflavin as was released would be available at a time when the products of digestion were on hand.

The remaining point is the failure of the animals on the level of 30 μg equivalents per gram of food to die sooner than they did. The only explanation appears to be that these two animals had the following history: they were made riboflavin deficient; used for the 9 μg of riboflavin group for 28 days, given 9 μg of riboflavin plus 9 μg equivalents of diethyl riboflavin for 21 days, deprived of flavin for 21 days to cause weight to reach a plateau and then placed on the 30 μg equivalents per gram diet. They may well have had better stores of riboflavin, but they did not gain as much weight as the other groups receiving the analog in the diet. This was very disconcerting until it was realized that these animals could very well have had relatively larger amounts of riboflavin available but could not use it successfully because of the more widely known type of inhibition; namely, because the diethyl analog reached a level in their blood where it was competing with the available riboflavin for the apoenzyme, or for the coenzyme-synthesizing enzymes.

This interpretation led directly to the inhibitor studies described above. It was felt essential for the support of the above theory that any group of animals receiving very large doses of the diethyl riboflavin must first show a tendency to grow and then very rapidly reach a plateau. As mentioned before, on the 5th day the animals of the 2 mg

diethyl riboflavin group had a highly significantly greater weight than the animals receiving no riboflavin. As a matter of fact, these animals were riboflavin-deficient on the 5th day, as evidenced by the fact that during the next 16 days the average gain was 2 gm, and during these 16 days 62% of the animals died. The animals on the riboflavin-deficient diet gained an average of 6 gm during the last 14 days of the test and, therefore, were not riboflavin-deficient by our criterion. All other groups receiving the diethyl riboflavin also showed this early weight increase.

If diethyl riboflavin is actually an inhibitor, it must be antagonized by riboflavin. We have shown that the process can be reversed in this way.

Two rather interesting observations were made in a search of the literature dealing with this matter of displacement of a vitamin from the tissues. What we thought to be a new idea certainly occurred first to Casimer Funk (Funk, '22). He stated, "We must not forget that the cure of acute beriberi symptoms may be accomplished theoretically in two ways, first, by the addition of the specific vitamin, second, by measures which will suddenly mobilize the reserve vitamins present in organs and tissues." We are not certain, but we believe the present study is the first support of Funk's second theoretical method of cure to be reported.

The other observation is that Kuhn (Kuhn et al., '37) may well have been dealing with the same phenomenon in his studies on 6,7-tetramethylene-9-(D-1'-arabityl)-isoalloxazine IX. This compound produced a very gradual weight increase in two riboflavin-deficient rats when administered at a level of 20 μg per day over a period of 10 weeks. The average gain during this time was about 15 gm, fully 10 gm of which were gained during the first two weeks. This was followed by essentially a plateau and during the last two weeks the animals lost weight. Ten micrograms per day led to death in from two to 5 weeks.

At present we are working out methods for the isolation of total flavin from the tissues of animals which have been

given diethyl riboflavin, and the separation of such preparations into riboflavin and diethyl riboflavin.

SUMMARY

Diethyl riboflavin has been found to possess approximately 90% of the activity of riboflavin for the bacterium *Bacillus lactis acidi.*

Diethyl riboflavin stimulates the growth of the riboflavin-deficient rat but cannot be considered as being capable of utilization in the place of riboflavin in the diet of this animal because it does not support life. Evidence is presented which suggests that this effect is due to a displacement mechanism.

Diethyl riboflavin has been found to be the most potent riboflavin inhibitor yet described, and the only riboflavin analog possessing vitamin properties for one type of organism and inhibitor properties for another.

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ADDENDUM

Since this report went to press we have found (D. W. Woolley, 1952, A Study of Antimetabolites, John Wiley and Sons, Inc., New York, p. 48) the following comment with reference to isoriboflavin made in a private communication from G. A. Emerson to D. W. Woolley. "The authenticity of isoriboflavin as an antimetabolite has been called in question by a recent observation that, whereas the original sample showed some effect in rats fed suboptimal riboflavin, subsequent samples exhibited no activity."

Several independently prepared lots of diethyl riboflavin were used for these studies. In the inhibitor portion of the work, several of the levels were repeated in different groups of animals with nearly identical results.

THE EXCRETION OF INJECTED P^{32} INTO THE GASTROINTESTINAL TRACT OF THE YOUNG CALF[1]

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THREE FIGURES

(Received for publication March 26, 1952)

In an earlier experiment (Lofgreen et al., '51) studies were made of the digestion and absorption of casein labeled with radioactive phosphorus, P^{32}, by the young calf. In order to study the extent to which casein is digested and to obtain a measure of the fecal protein and phosphorus which is of metabolic origin, the fecal excretion of P^{32} by calves fed labeled casein was studied. Since the gastrointestinal tract appears to play a major role in the excretion of phosphorus as well as of calcium (Forbes et al., '22; Bergeim, '26; Cowell, '37; Henry and Kon, '39), it appeared necessary to study the partition of the fecal P^{32} into that part which is absorbed and then re-excreted into the gut and that which is never absorbed. It is also important to know the time interval between feeding and the reappearance of measurable quantities of radioactive phosphorus in the feces. Lofgreen et al. ('51) found that the phosphorus in casein is probably absorbed in the inorganic form. If this is true, then it is possible, by frequent injections of labeled phosphate, to approximate closely the blood levels of P^{32} obtained when labeled casein is fed. Measurement of the radioactivity of fecal samples at various time intervals while these injections are be-

[1] This work was supported in part by a grant from the U. S. Atomic Energy Commission.

ing made makes it possible to estimate the time lag from initial injection to the first appearance of P^{32} from the blood and the rate of increase in its concentration. It appears that if the relationship between the P^{32} levels in blood and feces and the time from initial injection can be established, it may be possible to predict the amount of P^{32} being re-excreted when labeled casein is being fed. Such information would also add to knowledge of the phosphorus metabolism of the calf. Efforts to establish these relationships are reported in this paper.

TABLE 1

Description and treatment of calves

CALF NO.	BREED [1]	AGE	WEIGHT	DURATION OF EXPERIMENT	TOTAL P^{32} ADMINISTRATION
		days	*kg*	*hours*	*μc*
6	H	67	78.5	24	260
10	G	67	45.4	24	1,000
11	J	61	42.0	34	2,140
12	H	45	60.6	34	3,570

[1] Guernsey, Holstein and Jersey are abbreviated to G, H and J, respectively.

EXPERIMENTAL

In an effort to approximate as closely as possible the blood levels of P^{32} obtained in earlier trials after feeding labeled casein (Lofgreen et al., '51), 4 calves were injected with graded doses of P^{32} in an isotonic sodium phosphate solution every 20 minutes over a period of 24 or 34 hours. Blood samples were taken at frequent intervals throughout the experimental period. Injections were made into one jugular vein and samples were withdrawn from the other by means of venous catheterization similar to that described by Ralston et al. ('49). Fecal samples were obtained at approximately 4-hour intervals and at other times as the calves passed fecal material. The amount of P^{32} injected varied from 260 to 3,570 microcuries (μc). A summary of the description and treatment of the 4 calves is presented in table 1.

To obtain information on the excretion of phosphorus by the various segments of the gastrointestinal tract, the two calves, 6 and 10, were slaughtered immediately after a 24-hour injection period and samples were taken of the tissue and contents of the rumen, omasum, abomasum, jejunum, cecum, colon and rectum.

All samples were dried, mixed with magnesium nitrate and ashed at 500°C. and the ash taken up in HCl. The P^{32} content was determined by drying 0.2 ml of the ash solution on a copper planchet and counting the disintegrations with a Geiger counter. The method is described in more detail by Kleiber et al. ('51). Total phosphorus was determined by the method of Fiske and Subbarow ('25). The concentration of P^{32} is expressed as corrected specific activity, which is defined as the μc P^{32} per gram of phosphorus in any given sample divided by the μc P^{32} injected per 100 gm body weight.

RESULTS AND DISCUSSION

The blood and fecal levels are presented in figure 1. The blood levels shown do not exactly duplicate those obtained after casein feeding (Lofgreen et al., '51). The increase to a peak in from one to three hours observed in the blood levels of calves fed casein was not achieved in these injected calves. With this exception, however, the levels achieved by the two methods of administration were very similar.

With the dosages used no radioactivity was detected in the feces until approximately 5 hours after the initial injection. From the 5th hour the P^{32} concentration increased in a straight line relationship with time which can be described by the equation, $Y = 0.12X - 0.57$, where Y is the corrected specific activity of the re-excreted phosphorus and X is the time after initial injection between 5 and 34 hours. Since the phosphorus from casein is probably absorbed in the inorganic form, the injection of inorganic phosphate can be looked upon as representing the absorption of inorganic phosphate from the gastrointestinal tract into the blood stream. By use of the equation describing the increase in P^{32} con-

centration with time it is possible to calculate the corrected specific activity of the re-excreted phosphorus in the feces at any time after 5 hours from the initial injection.

It is interesting to note that even though the amount of P^{32} which comes from the re-excretion process is readily detectable, it is a very small proportion of the total P^{32} ex-

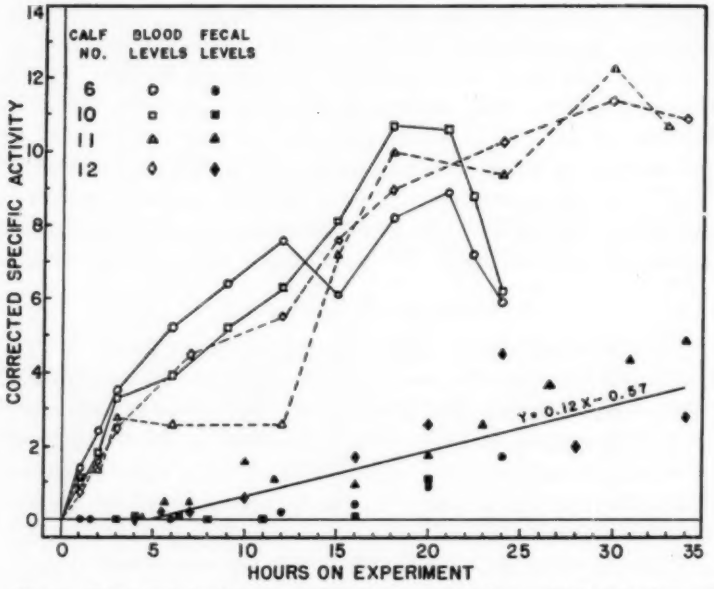

Fig. 1 Blood and fecal levels of P^{32} of calves receiving multiple injections of labeled phosphate solution.

creted when labeled casein is fed. For example, a fecal sample taken from a calf 24 hours after the first feeding of labeled casein had a corrected specific activity of 92.0 (unpublished data). The corrected specific activity of re-excreted phosphorus at 24 hours as calculated from the above equation is 2.3. Thus the correction for re-excretion amounts to only 2.5% of the total P^{32} which is excreted at this particular time. This, of course, does not represent the propor-

tion of the total phosphorus which is of metabolic origin, but only the P^{32} which is of metabolic origin at that time. The total metabolic phosphorus excretion will be discussed in another publication.

In figure 2 is presented the concentration of P^{32} in the tissues and contents of the gastrointestinal tract of the two calves which were slaughtered at the end of the 24-hour injection period.

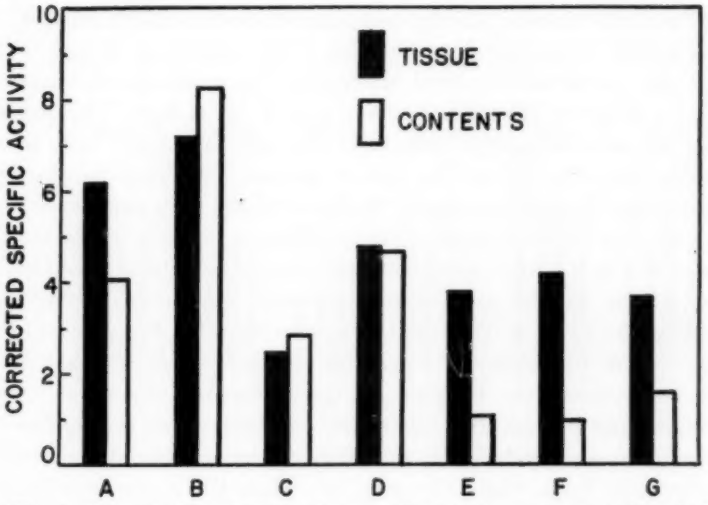

Fig. 2 Concentration of P^{32} in tissues and contents of the gastrointestinal tract of calves receiving multiple injections of labeled phosphate. A, rumen; B, omasum; C, abomasum; D, jejunum; E, cecum; F, colon; G, rectum.

The high concentration of P^{32} in the contents of the rumen, omasum, abomasum and jejunum in comparison to that in the cecum, colon and rectum is of particular interest. The concentration in the contents of the first 4 organs mentioned closely parallels that in the tissues, while the contents of the cecum, colon and rectum have a significantly lower concentration of P^{32} than their respective tissues. This suggests that the rumen, omasum, abomasum and jejunum may be

important in the excretion of phosphorus. In this connection it is interesting to note that the small intestine has been shown to play a predominant role in the excretion of calcium by the rat (Wallace et al., '51). Bergeim ('26) found a considerable excretion of phosphate into the small intestine of the rat.

The high activity of the contents of the rumen and omasum indicates that phosphorus readily passes from the blood into the contents of these two organs. The experiments of Wildt (1874) indicated that phosphorus excretion exceeded absorption in the rumen, while absorption exceeded excretion in the omasum. Scarisbrick and Ewer ('51), however, found that at any particular instant there may be a substantial movement of phosphate either into or out of the rumen. This could be the situation with respect to the omasum also. It is possible that the wall of the rumen and omasum may be merely a passive barrier through which phosphate may pass in either direction, depending upon the conditions existing in the blood and the gastrointestinal contents. The high rate of phosphate exchange of the rumen and omasum epithelium noted by Lofgreen et al. ('51) could be due merely to the passage of P^{32} from the blood through the epithelium to the gastrointestinal contents. Because of the numerous injections over a 24-hour period, the phosphate concentration in the blood would probably be such that the movement would be in the direction from blood to contents. Thus the concentration of P^{32} in the epithelium would increase due to the passage of the isotope through it. Obviously, the metabolism of the tissues of these two organs needs further study.

Since the calves received a practically continuous supply of P^{32} for the 24-hour period, it is interesting to compare these results with calves having received only one injection of the labeled phosphate. Unpublished data from this laboratory allow such a comparison. In figure 3 are presented the concentrations of P^{32} in the tissues and contents of the gastrointestinal tract of 4 calves, each of which received a single injection of P^{32}. At two, 5, 7, and 24 hours after in-

jection one of the 4 calves was slaughtered and samples taken for analysis.

At 24 hours the relation between the P^{32} content of the tissue and contents of the various organs was very similar to that presented in figure 2 for the calves receiving numerous injections. The only great difference was the lower concentration in the contents of the omasum of the calf having only one injection. The data show that the small intestine rapidly excretes P^{32} into the gastrointestinal tract, since at two hours after injection the P^{32} concentration was

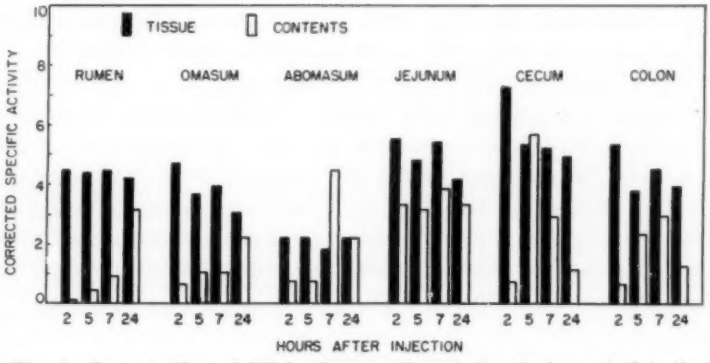

Fig. 3 Concentration of P^{32} in tissues and contents of the gastrointestinal tract of calves receiving one injection of labeled phosphate.

approximately equal to that at later times. The concentration of the isotope increases more slowly in the rumen, omasum and abomasum. This is a further indication that the small intestine is of predominating importance in the excretion of phosphorus. The increase in the specific activity of the contents of the cecum and colon at 5 and 7 hours is probably due to the passage along the tract of the active contents of the small intestine. The decrease in concentration from the 5th or 7th hour to the 24th hour in the contents of the cecum and colon despite the higher concentration in the corresponding tissues can be explained by one of two possibilities. If there were an influx of phosphorus of low

activity, as might possibly come from the plasma 24 hours after a single injection, this would dilute the P^{32} in the contents, causing a lowering of the specific activity. This seems improbable, however, since the tissues and contents of the cecum, colon and rectum from the multiple-injection calves showed the same general relationship at 24 hours, and in these calves the plasma concentration would still be high because of the numerous injections. The other possibilitity is that of selective absorption of the P^{32} from the contents of the cecum and colon. This is possible, since the phosphorus which passes through the gastrointestinal tract and reaches the cecum and colon is probably not available, while that being excreted into the contents by the upper segments of the tract may be in a form which allows it to be reabsorbed. Thus the phosphorus with a high specific activity would be absorbed, while the non-active phosphorus would remain in the tract, thus accounting for the decrease in specific activity. In this connection the data of Wallace et al. ('51) suggest that the cecum and colon of the young rat are concerned with reabsorption of calcium. Such may also be the case with phosphorus in the young calf.

SUMMARY

The time lag and the rate of excretion of injected P^{32} into the feces of young calves have been studied.

Under the conditions of this experiment no radioactivity could be detected until approximately 5 hours after the initial injection.

The rate of excretion of P^{32} into the feces can be calculated from the specific activity in the feces which can be expressed by the simple linear equation $Y = 0.12X - 0.7$, where Y is the corrected specific activity of the re-excreted phosphorus and X is the time after initial injection between 5 and 34 hours.

Data are presented which indicate that all segments of the gastrointestinal tract may play a role in the excretion

of phosphorus. The jejunum in particular appears to be important in this respect.

ACKNOWLEDGMENTS

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THE METABOLIC FECAL PHOSPHORUS EXCRETION OF THE YOUNG CALF[1]

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TWO FIGURES

(Received for publication March 24, 1952)

The problem of the partition of fecal phosphorus into that which has been absorbed and that which has not been absorbed is important for a number of reasons. Study of the availability of the phosphorus from various sources would be made easier if one had a reasonable estimate of the amount of the fecal phosphorus which is of metabolic origin. It is possible to determine the proportion of dietary phosphorus which is truly digested or absorbed by an animal when the metabolic phosphorus excretion is known. Furthermore, a knowledge of the metabolic fecal phosphorus excretion is important in the study of the over-all phosphorus metabolism of an animal. Since the principal pathway for the excretion of phosphorus is by way of the feces in certain animals (Forbes et al., '22; Henry and Kon, '39), the metabolic fecal phosphorus represents the major part of the total phosphorus loss from the body. It is possible that the daily maintenance requirement for phosphorus may have a close relationship with this daily phosphorus loss. Evidence for such a relationship between endogenous urinary nitrogen and the maintenance requirement for protein has been presented by Smuts ('35). Shohl ('39) points out that the fecal excretion of phosphorus increases when the ration consumed is alkaline

[1] This work was supported in part by a grant from the U. S. Atomic Energy Commission.

in nature. Since at the time of Shohl's review it was impossible to distinguish between phosphorus of metabolic origin and unabsorbed phosphorus, it could not be determined which fraction of the fecal phosphorus increased with the consumption of an alkaline diet. If the metabolic fecal phosphorus is dependent upon the type of diet being consumed, the daily maintenance requirement for phosphorus may not bear a close relationship to the daily metabolic loss. It may be, however, that the effect of an alkaline diet is upon the truly digested portion rather than the metabolic phosphorus. In either case the values for metabolic fecal phosphorus excretion obtained under a given set of conditions may be closely related to the maintenance requirement for phosphorus under these conditions.

The maintenance requirement for phosphorus of young growing calves is important in that it is one of the components of the total requirement. Any information on any one of the components of a given requirement facilitates determination of the total requirement through adding together its various parts. Such a procedure has been used for protein by Mitchell ('29) and Blaxter and Mitchell ('48) and for minerals by Mitchell and McClure ('37).

Metabolic fecal phosphorus can be measured by determining the phosphorus excreted on a phosphorus-free diet. Nicolaysen ('37) used this technique in his studies with rats. Such a procedure is open to criticism because of the abnormal conditions to which the animal must be subjected. Bosshardt and Barnes ('46) have shown, for example, that the values obtained for metabolic nitrogen excretion on a nitrogen-free diet are unreliable when applied to conditions of protein feeding. Hevesy ('48) used the tracer technique in measuring the metabolic phosphorus excretion in man by injecting radioactive phosphorus, P^{32}, and assuming that urinary phosphorus has the same specific activity as the blood plasma from which metabolic phosphorus originates. Kleiber et al. ('51) applied a modification of this method to the cow.

No information is available on the metabolic fecal phosphorus excretion of the young calf during the time it is being fed milk. The experiments reported in this paper were designed to make use of casein, a phosphorus-containing protein, as a tool in the determination of the metabolic fecal phosphorus of the young calf. Casein is, of course, a very important food for the calf and it would be interesting and of value

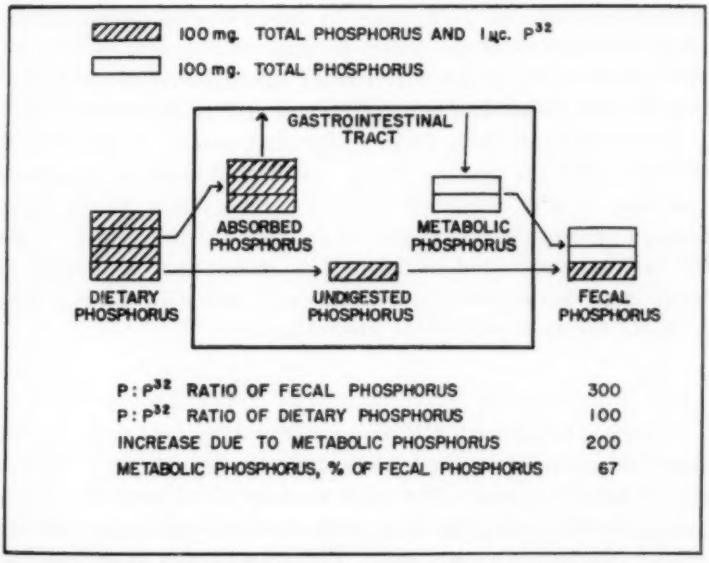
100 mg. TOTAL PHOSPHORUS AND 1 μc. P32
100 mg. TOTAL PHOSPHORUS
GASTROINTESTINAL TRACT
ABSORBED PHOSPHORUS
METABOLIC PHOSPHORUS
DIETARY PHOSPHORUS
UNDIGESTED PHOSPHORUS
FECAL PHOSPHORUS
P : P32 RATIO OF FECAL PHOSPHORUS 300
P : P32 RATIO OF DIETARY PHOSPHORUS 100
INCREASE DUE TO METABOLIC PHOSPHORUS 200
METABOLIC PHOSPHORUS, % OF FECAL PHOSPHORUS 67

Fig. 1 Principle of the method for the determination of metabolic fecal phosphorus by feeding P^{32} labeled casein.

to determine the extent to which the phosphorus in the casein molecule is utilized. Casein labeled with radioactive phosphorus, P^{32}, has previously been employed by Lofgreen et al. ('51) as a tracer in the study of the digestion and absorption of casein by the calf.

If casein labeled with P^{32} is fed to a calf, it is theoretically possible to determine the metabolic fecal phosphorus excretion according to the scheme shown in figure 1.

If we assume that the ratio of total phosphorus to radioactive phosphorus (P: P^{32} ratio) of the casein which passes through the gastrointestinal tract undigested is the same as that of the casein being fed, then any increase in this ratio is due to the passage of non-labeled body phosphorus into the feces. This method is based upon the assumption that the P^{32} content of the feces comes entirely from undigested casein. Since it has been found that absorbed radioactive phosphorus begins to appear in the feces of young calves approximately 5 hours after feeding (Lofgreen et al., '51, '52), the feces samples taken after labeled casein is fed will contain P^{32} which has been absorbed and re-excreted as well as that coming directly from undigested casein. It was found, however, that the amount being re-excreted could be predicted and that it was relatively small in proportion to the total isotope present in the feces. It is possible, therefore, to use the method presented in figure 1 to determine the metabolic fecal phosphorus excretion of the calf provided a correction is made for re-excretion of absorbed P^{32}.

EXPERIMENTAL PROCEDURES

Casein labeled with P^{32} was produced by injecting an isotonic solution of sodium phosphate containing the isotope into a lactating cow. The milk was collected and the casein prepared according to the method described in an earlier report (Lofgreen et al., '51). This casein was then incorporated as the sole source of protein and of phosphorus into a purified liquid diet similar to that described by Wiese et al. ('47). Four calves were fed a purified diet containing unlabeled casein from two to three days of age until they were used on these experiments. They were then given either one or two feedings of the diet containing the labeled casein. Blood and fecal samples were collected periodically for 24 to 36 hours after the initial feeding of the labeled casein. A summary of the information concerning the calves and their treatment is given in table 1.

A small amount of carmine was mixed with the diet at the first feeding of the labeled casein. Lampblack was added to the diet at the second feeding whether the calves were fed unlabeled or labeled casein. The addition of these two substances was for the purpose of indicating when the feces being excreted corresponded to the feeding of the labeled casein. Fecal samples were taken from the calves given one feeding of labeled casein only when the carmine began to appear. The entire fecal excretion of the calves given two feedings of the labeled casein was collected, each defecation being collected separately. Sampling was continued in both cases until lampblack began to appear in the feces. The P^{32} content of each sample was determined separately, while a composite was made for determination of total phosphorus excretion. Radioactivity was determined upon the fresh as well as the ashed samples. The P^{32} content is expressed as the corrected specific activity, which is equal to the microcuries (μc) P^{32} per gram of total phosphorus divided by the dose (in μc) per 100 gm body weight.

TABLE 1

Description and treatment of calves

CALF NO.	BREED	AGE	WEIGHT	FEEDINGS OF LABELED CASEIN	TOTAL P^{32} ADMINISTERED
		days	*kg*		*μc*
5	Holstein	63	57.2	1	26.5
7	Holstein	68	68.5	1	72.0
13	Jersey	25	27.0	2	22.1
14	Holstein	14	36.0	2	29.5

RESULTS AND DISCUSSION

The calculation of the metabolic fecal phosphorus according to the method described is presented in table 2.

The fecal sample used for the determination was the last sample containing the carmine in the case of the two calves given one feeding of labeled casein, while the first sample

containing the lampblack was used in the case of those given labeled casein at two successive feedings. Thus in the latter case the fecal sample corresponded to the second feeding of labeled casein. If there were any mixing of stomach contents, the estimate of the metabolic fecal phosphorus based upon a sample of feces corresponding to the second feeding

TABLE 2

The metabolic fecal phosphorus of calves fed P^{32}-labeled casein

CATEGORY OF INTEREST	CALF NO.			
	5	7	13	14
Time of sampling feces, hours after initial feeding	18	18	30	30
Corrected specific activity of feces containing re-excreted P^{32}	42.8	54.3	122.0	118.0
Corrected specific activity of re-excreted P^{32} [1]	1.6	1.6	3.0	3.0
Corrected specific activity of feces minus re-excreted P^{32}	41.2	52.7	119.0	115.0
Dose of P^{32}, μc per 100 gm body weight	0.046	0.105	0.082	0.082
Specific activity of feces [2]	1.9	5.5	9.7	9.4
P:P^{32} ratio of feces [3]	526	182	103	106
P:P^{32} ratio of casein fed [3]	159	69	27	27
Increase in ratio due to metabolic P	367	113	76	79
Metabolic phosphorus:				
% of fecal P	70	62	74	75
mg per day	..	..	117	152
mg per kg body weight	..	..	4.3	4.2
mg per 100 gm dry matter intake	..	..	33.3	32.4

[1] Calculated from equation: $Y = 0.12X - 0.57$ (Lofgreen et al., '52).
[2] Corrected specific activity × dose of P^{32} per 100 gm body weight.
[3] Reciprocal of specific activity × 1000.

would be more reliable than one corresponding to the first feeding of labeled casein. For example, if the last fecal samples containing carmine, and thus corresponding to the first feeding of labeled casein to calves 13 and 14, are used to calculate the metabolic phosphorus, values of 80 and 77% of the fecal phosphorus are obtained, while those determined from samples corresponding to the second feeding are 74 and 75%, respectively.

If the metabolic fecal phosphorus excretion of the calf makes up the major portion of the total phosphorus loss from the body, and if this loss has a close relationship to the maintenance requirement for phosphorus, it is then possible to calculate the theoretical maintenance requirement of the calf from its metabolic fecal phosphorus excretion. Henry and Kon ('39) found that 90% of the phosphorus lost by rats on a milk diet was in the feces. Forbes et al. ('22) and Kleiber et al. ('51) found that in cattle the phosphorus excretion in the urine amounted to less than 1% of that excreted by way of the feces. If we assume that the 4.3 and 4.2 mg metabolic

TABLE 3

The apparent and true digestibility of the phosphorus of casein

CATEGORY OF INTEREST	CALF NO.	
	13	14
Total phosphorus intake, mg per day	607	810
Total fecal phosphorus, mg per day	158	202
Metabolic fecal phosphorus, mg per day	117	152
Phosphorus apparently digested, mg per day	449	608
Phosphorus truly digested, mg per day	566	760
Apparent digestibility of phosphorus, %	74	75
True digestibility of phosphorus, %	93	94

phosphorus per kilogram body weight excreted by calves 13 and 14, respectively, represent approximately 90% of the total loss, then the net amounts required to replace the total loss would be 4.8 and 4.7 mg, respectively. This would amount to approximately 0.22 gm phosphorus per day for a 100-pound calf. If a 50% utilization is assumed, the dietary requirement to replace the phosphorus loss would be 0.44 gm per day. According to Morrison ('48), the daily phosphorus requirement of a 100-pound growing calf is 6 gm. Of this amount, approximately 7.5% would be necessary to replace the metabolic loss.

The apparent and true digestibilities of the phosphorus in casein are presented in table 3.

These data reveal that the phosphate in the casein molecule is readily available to the calf. It is interesting to note that the truly digested phosphorus from the casein consumed by each calf is approximately 5 times the amount excreted as metabolic fecal phosphorus.

Kleiber and associates ('51) determined the metabolic fecal phosphorus in the dairy cow by injecting P^{32} and assuming that the specific activity of the plasma is the same as that of the metabolic fecal phosphorus. Since frequent blood samples were taken from calves 13 and 14, it was possible to use a modification of this method of calculating the metabolic fecal phosphorus and to compare its results with the values obtained by use of the $P : P^{32}$ ratio of the feces and casein. If the metabolic fecal phosphorus has the same specific activity as the plasma, and the remainder of the fecal phosphorus comes from undigested casein, the specific activity of the feces can be represented as:

$$\phi = \pi q + \gamma(1 - q), \qquad (1)$$

where ϕ, π and γ are the specific activities of the feces, plasma and casein respectively, and q is the proportion of the fecal phosphorus which has the same specific activity as the plasma and thus represents that which is of metabolic origin. Since we can measure ϕ, π and γ, it is possible to calculate q. In earlier trials (Lofgreen et al., '52) it was found that injected P^{32} began to appear in the feces approximately 5 hours after initial injection. The plasma level 5 hours prior to the time the fecal sample was taken is therefore used in these calculations. In figure 2 are presented the plasma levels of calf 13 at various times after the initial feeding of casein. From the 8th to the 30th hour the plasma specific activity can be expressed as a linear function of time and can be described by the equation:

$$\pi = 0.23\,t + 20.4, \qquad (2)$$

where π is the corrected specific activity of the plasma and t is the time in hours after initial feeding. The fecal sample taken from calf 13 at 30 hours after initial feeding had a

corrected specific activity of 122, the plasma 5 hours earlier had a corrected specific activity of 26, and the corrected specific activity of the casein was 446. By substitution of these values in equation (1), a value of 0.77 or 77% is obtained for q, which is equal to the proportion of the fecal phosphorus which is of metabolic origin. This is not far different from

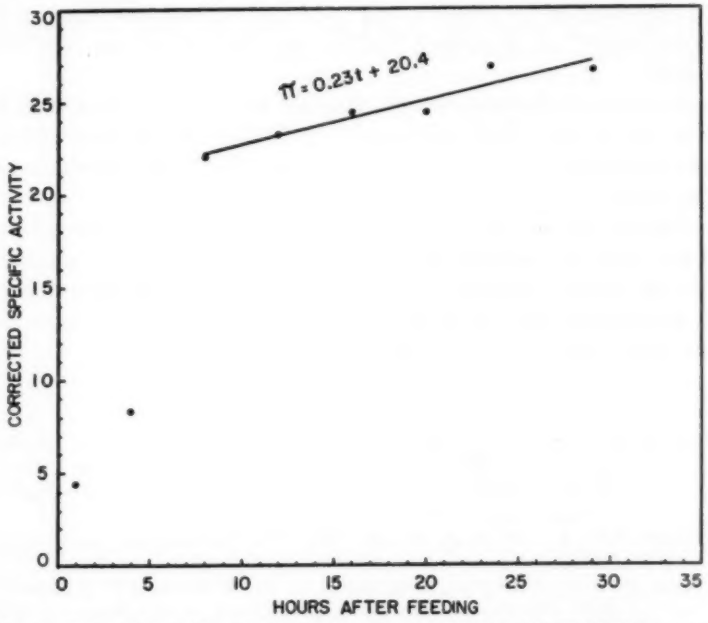

Fig. 2 Corrected specific activity of the plasma of calf 13 fed P^{32}-labeled casein.

the value of 74% obtained on the same fecal sample by the $P : P^{32}$ ratio in feces and casein. For calf 14 values obtained by the two methods were 78 and 75%, respectively. The values obtained by the two methods are therefore in good agreement and provide evidence of the suitability of the method described for determination of the metabolic fecal phosphorus of the young calf. The agreement between the two methods

also lends support to the assumption that the metabolic fecal phosphorus has the same specific activity as the blood plasma.

SUMMARY

A method is presented for the estimation of the metabolic fecal phosphorus excretion of the young calf by feeding casein labeled with P^{32} in a liquid diet. Values obtained by this method on two calves were 4.3 and 4.2 mg per kilogram body weight, or 33.3 and 32.4 mg per 100 gm of dry matter intake.

The true digestibility of the phosphorus of casein was found to be about 93%, and the amount truly digested by two calves was approximately 5 times the metabolic fecal phosphorus excretion.

Values for the metabolic fecal phosphorus excretion calculated from the specific activity of the casein and the plasma specific activity 5 hours prior to the sampling of the feces were in good agreement with those obtained from the difference in the ratios of $P : P^{32}$ in feces and casein.

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TRI-O-CRESYL PHOSPHATE AS A VITAMIN E ANTAGONIST FOR THE RAT AND LAMB [1]

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FOUR FIGURES

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In recent years vitamin E deficiency has been associated with certain diseases of domestic ruminants fed practical rations. Thus, Willman and co-workers ('45) reported that the occurrence of "stiff-lamb disease" could be prevented by administration of mixed tocopherols either prepartum to the ewes or postpartum to the newborn lambs. Gullickson and Calverley ('46) found that mature cattle maintained on tocopherol-low rations over a period of several years were prone to sudden death under circumstances suggesting cardiac failure, a diagnosis supported by previous observance of abnormal electrocardiograms and by atrophy of cardiac fibers.

On the other hand, vitamin E deficiency symptoms in the laboratory rat are commonly not discernible prior to the occurrence of reproductive failure. The hemolysis test of Rose and György ('50), founded on the observation that tocopherol-deficient erythrocytes hemolyze in a dilute solution of buffered dialuric acid while normal cells do not, has provided a useful tool in detecting an early deficiency in this species.

That oral administration of tri-o-cresyl phosphate to rabbits quickly induces symptoms of vitamin E deficiency was reported by Meunier, Vinet and Jouanneteau ('47), and

[1] Presented before the April, 1952, meeting of the American Institute of Nutrition, New York, New York. Fed. Proc., *11:* 204 (1952).

Meunier and Chenavier ('49) ascribed a similar effect to di-o-cresyl succinate. The toxicity of both compounds was shown to be reversible by tocopherol administration.

In the present experiment the results of maintaining rats and newborn lambs on tocopherol-low diets, with and without the inclusion of tri-o-cresyl phosphate, are reported.

TABLE 1

Composition of basal diet for rats

CONSTITUENTS	CONCENTRATION
	%
Cerelose	32.75
Casein ("vitamin-free")	30
Tocopherol-low lard	30
Salt mixture [1]	4
Sulfathalidine	2
Choline drymix	0.40
Vitamin premix [2]	0.75
Vitamin A and D concentrate (100,000 A; 10,000 D)	0.10

[1] Salts 446. See Spector ('48).

[2] To provide the following concentrations of vitamins (micrograms per gram of diet): thiamine 3.75, riboflavin 3.75, nicotinic acid 7.5, pyridoxine 3.75, calcium pantothenate 15.0, biotin 0.075, folic acid 0.75 and 2-methyl-1,4-naphthoquinone 0.75.

EXPERIMENTAL

Part I. Rat experiment

Three groups of 8 and 4 groups of 6 weanling male rats of the Sprague-Dawley strain, averaging about 50 gm in weight, were employed. They were housed and fed individually.

A basal diet was used in which alpha-tocopherol was omitted and tocopherol-low lard,[2] produced by molecular distillation, served as a source of fat. The composition of this diet is given in table 1. Additional amounts of vitamin A were administered orally.

[2] Supplied by Distillation Products, Inc., Rochester 13, New York, through the courtesy of Dr. P. L. Harris.

The 7 groups of rats were assigned to the following treatments:

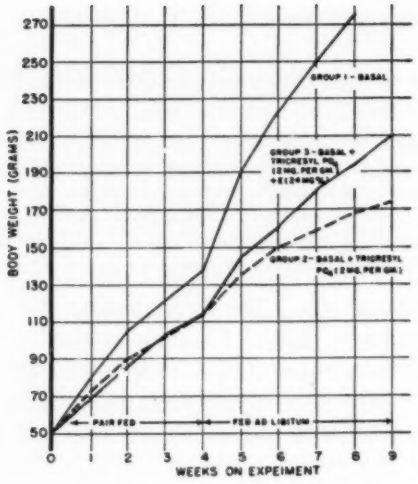
Groups 1 and 5 — basal
Groups 2 and 6 — basal + tri-o-cresyl phosphate (2 mg/gm of diet)
Group 3 — basal + tri-o-cresyl phosphate (2 mg/gm of diet) + DL-alpha-tocopherol acetate (0.24 mg/gm of diet)
Group 4 — basal + DL-alpha-tocopherol acetate (0.24 mg/gm of diet)
Group 7 — basal + tri-o-cresyl phosphate (6 mg/gm of diet).

Results and discussion. The growth data for the various groups are plotted in figure 1 and a summary of average daily gains and food consumption is given in table 2.

Because the palatability of the diets containing tri-o-cresyl phosphate was obviously impaired, groups 1, 2 and 3 were pair-fed during the first 4 weeks of the experiment. At this time it became evident that group 3, receiving the antagonist plus vitamin E, was being limited in food intake, and hence in growth rate, by group 2, receiving the tri-o-cresyl phosphate supplement alone. Subsequently these groups were fed ad libitum and an immediate differential in growth rates developed. Group 1 rats, maintained on the basal diet, grew at substantially faster rates than those of the other two groups even when pair-fed, a result possibly attributable to a mild diarrhea in the animals receiving tri-o-cresyl phosphate.

That the rats of group 2 were deficient in vitamin E was confirmed by the hemolysis test of Rose and György ('50). Red blood cells from these animals hemolyzed in a dilute solution of dialuric acid in 30 to 120 minutes (average, 62 minutes), indicating a mild deficiency. Erythrocytes from 4 rats of group 6, fed the same tri-o-cresyl phosphate-supplemented basal diet, showed complete hemolysis after 4 hours, while those of the two others showed partial hemolysis. Cells from group 3, receiving the antagonist plus vitamin E, failed to hemolyze through 24 hours' observation, and those of group 5 rats, maintained on the basal regimen for 10 weeks, hemolyzed in 6 to 9 hours, denoting a very slight deficiency.

Rats fed tri-o-cresyl phosphate at a level of 2 mg per gram of diet developed a rough haircoat in two to three weeks,



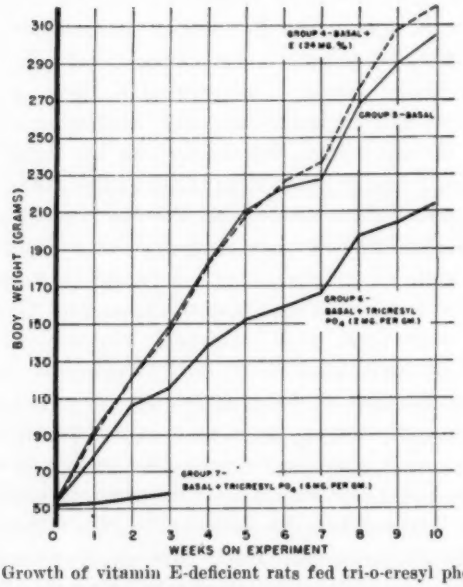

BODY WEIGHT (GRAMS)
WEEKS ON EXPERIMENT
GROUP 4 - BASAL + E (24 MG. %)
GROUP 5 - BASAL
GROUP 6 - BASAL + TRICRESYL PO4 (2 MG. PER GM.)
GROUP 7 - BASAL + TRICRESYL PO4 (6 MG. PER GM.)

Fig. 1 Growth of vitamin E-deficient rats fed tri-o-cresyl phosphate.

Upper chart: Group 1 — basal (E-free)
Group 2 — basal + tri-o-cresyl phosphate (2 mg/gm of diet)
Group 3 — basal + tri-o-cresyl phosphate (2 mg/gm of diet) + DL-alpha-tocopherol acetate (0.24 mg/gm of diet)

Lower chart: Group 4 — basal + vitamin E
Group 5 — basal (E-free)
Group 6 — basal + tri-o-cresyl phosphate (2 mg/gm of diet)
Group 7 — basal + tri-o-cresyl phosphate (6 mg/gm of diet)

which in some cases became very severe. The concentration of alpha-tocopherol present in the diet of group 3 (24 mg) was inadequate to protect most of the rats against this condition, and in these cases marked improvement in appearance and appetite was observed following oral administration of vitamin E. Tri-o-cresyl phosphate at a level of 6 mg per gram of diet was highly toxic for the rats of group 7, and all these animals died during the third or 4th weeks of the experi-

TABLE 2

Average daily gains and food consumption of tri-o-cresyl phosphate-treated and control rats

GROUP	NO. RATS	DIET	WEEKS ON DIET	AVERAGE DAILY GAIN	AVERAGE DAILY FOOD CONSUMED
				gm	*gm*
1	8	Basal	8	3.76 ± 0.303	7.67 ± 0.38
2	8	Basal + tri-o-cresyl PO_4 [1]	9	1.98 ± 0.179 [4]	6.80 ± 0.40
3	8	Basal + tri-o-cresyl PO_4 + E [2]	9	2.54 ± 0.179 [4]	7.99 ± 0.20
4	6	Basal + E [3]	10	3.84 ± 0.078	10.51 ± 0.11
5	6	Basal	10	3.61 ± 0.574	10.77 ± 0.025
6	6	Basal + tri-o-cresyl PO_4 [1]	10	2.32 ± 0.174	8.32 ± 0.36
7	6	Basal + tri-o-cresyl PO_4 [3]	3	0.27 ± 0.177	3.81 ± 0.00

[1] Two milligrams per gram.
[2] Twenty-four milligrams per cent.
[3] Six milligrams per gram.
[4] Group 3 vs. group 2 significant ($P = 0.024$).

ment after exhibiting extreme roughness of fur and emaciation.

The results of this experiment demonstrate that tri-o-cresyl phosphate in the diet of the rat has an antagonistic effect on vitamin E, as evidenced by reduced growth rate and hemolysis of red blood cells in dilute dialuric acid solution.

Part II. Lamb experiment

Twelve lambs, one to 5 days of age, were used in a second experiment designed to induce a vitamin E deficiency by feeding a tocopherol-low synthetic milk diet, with and with-

out the addition of tri-o-cresyl phosphate. The lambs were housed in metabolism cages and were fed ad libitum. The composition of the experimental diet is given in table 3.

The synthetic milk was compounded as described previously by Draper and Johnson ('51) so as to contain 19.5% total solids. Vitamins A and D were provided in aqueous emulsion and the remaining vitamins, including 2-methyl-1,4-

TABLE 3

Composition of synthetic milk basal diet

CONSTITUENTS	CONCENTRATION
Casein "vitamin-free" (%, dry basis)	30
Lard, tocopherol-low (%, dry basis)	30
Mineral mixture[1] (%, dry basis)	6
Cerelose (%, dry basis)	34
Water-soluble vitamins (mg per liter of diet)	
Thiamine hydrochloride	0.65
Riboflavin	1.30
Calcium pantothenate	1.95
Nicotinic acid	2.60
Pyridamine	0.65
Choline	260.0
Folic acid	0.26
Biotin	0.02
Fat-soluble vitamins (quantity per liter of diet)	
Vitamin A	2,000 I.U.
Vitamin D	200 I.U.
2-methyl-1,4-naphthoquinone	0.26 mg

[1] For composition of mineral mixture see Johnson, James and Krider ('48).

naphthoquinone, were added to the milk in a 30% ethanol solution. Vitamin A was also given individually to all lambs at a rate of 25,000 units per week. Sulfathalidine was included at a level of 3 gm per liter of diet.

The lambs were grouped with respect to dietary treatment as follows:

Group 1 (lambs 1 to 5) — basal + tri-o-cresyl phosphate
Group 2 (lambs 6 to 9) — basal
Group 3 (lambs 10 to 12) — basal + tri-o-cresyl phosphate + DL-alpha-tocopherol acetate.

Disposition of lambs. The lambs of group 1 grew normally and appeared in good health after three weeks on the basal diet supplemented with tri-o-cresyl phosphate at a level of 400 mg per liter (approximately 2 mg per gram dry matter). At this time the concentration of the antagonist was increased to 1 gm per liter of diet. Subsequently, the effects of this regimen were manifested in two ways, either by sudden death or by development of a paralysis of the legs accompanied by hyperthermia and followed by death in one to three days. Two of the lambs (1 and 4) succumbed suddenly on the 30th day of the experiment. One of the remaining three in this group (number 5) exhibited paresis on the 24th day at which time a rectal temperature of 108°F. was recorded. Two hundred and fifty milligrams of DL-alpha-tocopherol were administered orally, along with subcutaneous penicillin and isotonic glucose, but the lamb died about 36 hours after first showing symptoms. A similar series of observations was made on lambs 2 and 3, beginning on the 30th day of the experiment. Paresis appeared suddenly, primarily affecting the hind legs, which became ineffectual in supporting the weight of the animals. Reversal of these symptoms could not be obtained with tocopherol administered orally, even though tri-o-cresyl phosphate was withdrawn from the diet and, in the presence of anorexia, the animals were fed by stomach tube.

The lambs of group 2, fed the basal diet without the antagonist, did not develop severe signs of deficiency until approximately 7 weeks had elapsed. At this time lamb 6 exhibited diarrhea and anorexia, succumbing on the 54th day without showing evidence of paresis. Lamb 7 developed a leg weakness on the 55th day similar to that described for three of the lambs in group 1 (see fig. 2). This animal was maintained by stomach tube feeding over a period of 12 days during which alpha-tocopherol was administered. In addition to the vitamin E given orally, an emulsion of tocopherol and Tween 80 in isotonic glucose was given intravenously in an amount

calculated to provide 120 mg of the vitamin, but no recovery of coordination was obtained.

Lamb 8 exhibited anorexia and emaciation after 55 days on the basal diet, at which time alpha-tocopherol was administered orally (300 mg every 4 days) and intravenously as a single dose of 120 mg. Beginning 10 days after the initial treatment, this lamb improved remarkably in appearance and appetite, and gained an average of 0.46 lb. per day over the next two weeks. The 4th lamb in this group, number 9, failed to show any serious ill effects of the deficient regimen

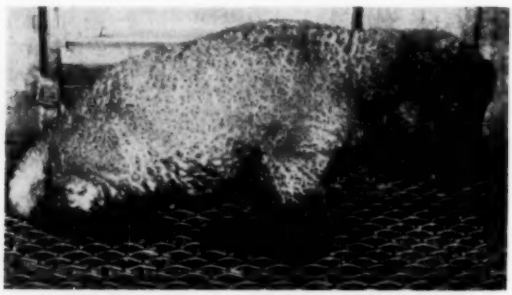


Fig. 2 Vitamin E-deficient lamb. Lamb 7 after 55 days on the vitamin E-free "synthetic milk" ration. (Note the leg weakness and hind-leg paralysis.)

after 12 weeks, though growth was very poor during the last 4 weeks of this period. The resistance of this animal may be attributable to the fact that it received colostrum for 5 days following birth from a dam presumably receiving adequate amounts of vitamin E.

The evidence that tri-o-cresyl phosphate exerted a deleterious effect on vitamin E metabolism in the lambs of group 1 was supported by the performance of the group 3 animals (10, 11 and 12) which received tocopherol as well as tri-o-cresyl phosphate in the diet. During the first 4 weeks alpha-tocopherol was provided in a single weekly dose of 100 mg, but at this time a slight incoordination developed in lamb 11 and the dose to all three was subsequently increased to 500 mg

per week. A similar condition was observed in lamb 12 after 40 days, though in both animals the symptoms were very mild. The third member of this group grew rapidly and remained free of deficiency symptoms for 10 weeks. Thus, the addition of alpha-tocopherol to the tri-o-cresyl phosphate-supplemented diet delayed or eliminated the symptoms observed in group 2. Judging from the failure of the vitamin completely to protect two of the lambs from paresis at the level used in this experiment, the inhibition ratio of tri-o-

TABLE 4

Growth rates and food consumption of vitamin E-deficient and control lambs

LAMB NO.	DIET	DAYS ON DIET	AVE. DAILY GAIN	AVE. DAILY FOOD CONSUMED
			lb.	*lb.*
1	Basal + tri-o-cresyl PO_4	30	0.27	0.37
2	Basal + tri-o-cresyl PO_4	30	0.48	0.61
3	Basal + tri-o-cresyl PO_4	30	0.30	0.41
4	Basal + tri-o-cresyl PO_4	30	0.54	0.67
5	Basal + tri-o-cresyl PO_4	24	0.55	0.73
6	Basal	54	0.36	0.65
7	Basal	55	0.19	0.40
8	Basal	55	0.11	0.40
9	Basal	77	0.40	0.60
10	Basal + tri-o-cresyl PO_4 + E	70	0.53	0.67
11	Basal + tri-o-cresyl PO_4 + E	42	0.35	0.53
12	Basal + tri-o-cresyl PO_4 + E	42	0.36	0.60

cresyl phosphate to vitamin E appears to be relatively narrow, at least in this species.

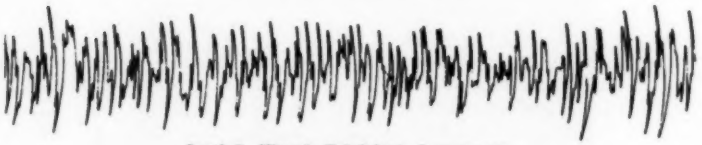
The average daily gains and food consumption of the lambs are shown in table 4.

Electrocardiograms. Electrocardiograms of 4 lambs were recorded, including two deficient animals and two healthy controls of comparable age which had been fed a good-quality practical ration. At the time of the recordings, lamb 7 was exhibiting severe paralysis after 55 days on the basal diet, and lamb 8 showed emaciation and weakness after a similar period. Representative sections from these records are shown in figure 3.

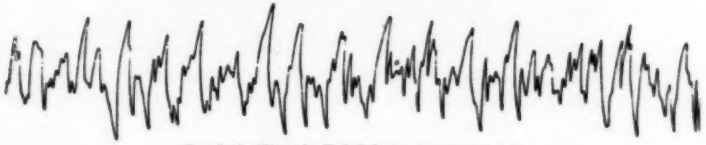
The most striking feature of the electrocardiograms is the erratic record obtained with lamb 7, which appears grossly abnormal when compared to those of the control animals (21 and 28). The electrocardiogram for lamb 8 features a

Lamb 21. Control. 5 cm per sec.

Lamb 28. Control. 10 cm per sec.



Lamb 7. Vitamin E-deficient. 5 cm per sec.



Lamb 7. Vitamin E-deficient. 10 cm per sec.

Lamb 8. Vitamin E-deficient. 5 cm per sec.

Fig. 3 Electrocardiograms of vitamin E-deficient and control lambs. All leads left foreleg-right foreleg.

depolarization preceding the R wave which is not present in either of the controls. The deficient animals do not show a decrease in over-all potential as reported by Gullickson and Calverley ('46) for a vitamin E-deficient cow, though the record for lamb 7 denotes a more profound disturbance. The

observations on the two deficient lambs indicate that the abnormalities are more severe in animals exhibiting paresis, and support the finding of Bragdon and Levine ('49), who reported that disturbances in the electrocardiograms of vitamin E-deficient rabbits occur only after muscular dystrophy is well advanced. The circumstances surrounding the death of two of the lambs receiving tri-o-cresyl phosphate in this experiment, when interpreted in the light of the electrocardiograms of other deficient animals, indicate that death was due to cardiac failure.

Creatine and creatinine excretion. Using the method of Folin ('14), creatine and creatinine were determined in the urine of the lambs at the beginning of the experiment and at intervals thereafter. The average creatinine coefficient (milligrams creatinine excreted per kilogram body weight per day) for the lambs at the start of the trial was 21.6 mg, with a standard deviation of 3.1 mg, a value comparable to that for man but appreciably greater than that of 11.18 reported by Dinning, Gallup and Briggs ('49) for beef steers. The coefficients tended to decline in lambs fed the tocopherol-low diet, the average value for the 8 lambs after three weeks being 18.4 ± 4.7 mg, and those of lambs 2 and 3 when exhibiting paresis being only 5.1 and 7.2 mg, respectively.

Creatine coefficients for deficient animals increased, as did the creatinine ratios ("total" creatinine divided by preformed creatinine, where "total" creatinine represents creatine excreted in addition to preformed creatinine). Initial ratios for the 8 lambs on the deficient diet ranged from 1.03 to 2.07 with a mean of 1.60. Values obtained for 5 paralyzed lambs were 5.61, 3.86, 8.09, 4.08 and 3.11.

Histology of skeletal muscle. Samples of muscle taken from the heavily muscled areas of the hind limbs were examined histologically. The tissues were fixed in Bouin's solution, sectioned by the usual paraffin procedure at a thickness of 8 μ, and stained with Harris' hematoxylin and eosin.

Histological degeneration of the striated muscles of all the lambs receiving the tri-o-cresyl phosphate-supplemented basal

diet may be arbitrarily classified as severe to very severe. Degeneration of fibers was widespread, fibrosis and proliferation of nuclei were evident and large areas of the sections were devoid of any normal fibers, their place being taken by connective tissue. Fatty infiltration and phagocytosis were common.

Muscle sections from the lambs on the basal diet only showed a milder, but definite, type of degeneration in some areas. Moderate degeneration was indicated by unevenness of fibers, early evidence of nuclear proliferation and hyalination of some fibers.

Tissue from the lambs of group 3, treated with both tri-o-cresyl phosphate and vitamin E, appeared normal in all cases. Photomicrographs of representative sections of skeletal muscle from the three groups of lambs are shown in figure 4.

Plasma tocopherol levels. Plasma tocopherol determinations were conducted in duplicate according to the method of Quaife and Biehler ('45). Initial values ranged from 0.18 to 0.37 mg of tocopherols per 100 ml plasma, with a mean of 0.27 mg. Tocopherol levels in the plasma of three lambs exhibiting paralysis after 30 days on the basal diet containing tri-o-cresyl phosphate were 0.12, 0.08 and 0.09 mg %, respectively. Values determined for two lambs on the basal diet shortly before death were 0.05 and 0.07 mg %. Corresponding values for the lambs given tri-o-cresyl phosphate in the diet in addition to large amounts of alpha-tocopherol for 6 weeks were 0.43, 0.39 and 0.33 mg %, respectively.

The outward manifestations of vitamin E deficiency observed in the lambs fed a tocopherol-low diet, either with or without the addition of tri-o-cresyl phosphate, together with the concomitant occurrence of muscular dystrophy, creatinuria and low plasma tocopherol levels, indicate that this syndrome is identical with "stiff-lamb disease," for which similar observations have been reported (Willman and co-workers, '40, '45). A similar conclusion was reached by Culik et al. ('51) in a study recently reported.

SUMMARY AND CONCLUSIONS

Newborn lambs were fed a purified "synthetic milk" diet deficient in vitamin E and containing tocopherol-low molecular-distilled lard as a source of fat. Those animals maintained on this basal diet for about 7 weeks exhibited either paralysis of the legs or anorexia and weight loss. Addition of tri-o-cresyl phosphate to the basal diet (0.4 to 1.0 gm per liter of "milk" containing 19.5% dry matter) caused either severe leg weakness or sudden death after approximately 4 weeks. The effects of this compound could be delayed or prevented, but not cured, by oral administration of synthetic DL-alpha-tocopherol acetate at a rate of 100 mg per week. Lambs showing paresis induced by tri-o-cresyl phosphate ingestion died within three days despite the administration of large amounts of tocopherol either orally or intravenously as an emulsion. The outward manifestations of vitamin E deficiency obtained by feeding this diet, in addition to the muscular dystrophy, creatinuria and low plasma tocopherol levels also observed, indicate that the syndrome is identical with "stiff-lamb disease" which occurs among the suckling offspring of ewes fed a vitamin E-low practical ration. Abnormalities in the electrocardiograms of paralytic lambs were also detected. In a corollary experiment, it was found that when the basal diet was fed to rats, the addition of tri-o-cresyl phosphate (2 mg per gram) induced earlier development of a vitamin E deficiency, as identified by growth depression and the hemolysis test of Rose and György ('50), than was obtained on the basal regimen alone. Supplementation with synthetic DL-alpha-tocopherol acetate (24 mg per 100 gm diet) prevented the occurrence of deficiency symptoms in rats receiving the above level of tri-o-cresyl phosphate in the diet over a 10-week period.

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PLATE 1

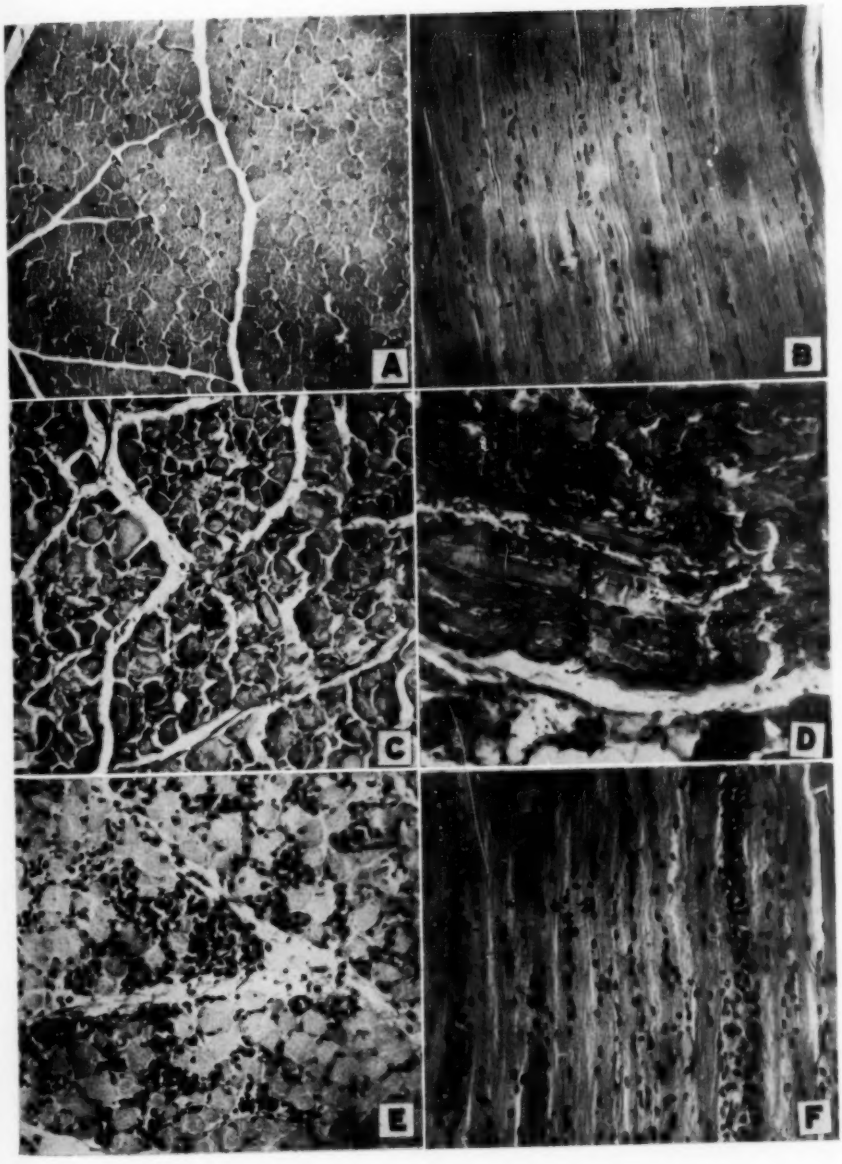
EXPLANATION OF FIGURES

4 Photomicrographs of sections of striated muscle from experimental lambs. Magnification × 108 (original × 144 reduced one-fourth). A, C, E cross sections. B, D, F longitudinal sections.

A and B: lambs received basal ration + tri-o-cresyl phosphate + DL-alpha-tocopherol acetate. Muscle appeared normal.

C and D: lambs received basal E-deficient ration + tri-o-cresyl phosphate. Note necrosis of muscle fibers, myophagocytosis and fatty infiltration.

E and F: lambs received basal E-deficient ration only. Most of the muscle appeared normal, but areas as shown with beginning dystrophy were found. Note the proliferation of nuclei, myophagocytosis and beginning hyalinization of some fibers.


A
B
C
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E
F

COMPARISON OF METHODS FOR DETERMINATION OF THE VITAMIN B_{12} POTENCY OF MEATS[1]

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ONE FIGURE

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The importance of evaluating the nutrient content of foods with animal assays as compared to alternate methods of analysis (i.e., chemical or microbiological) is well recognized. These studies have been particularly useful in establishing the specificity of different analytical methods in terms of the animal assay and in affording evidence for the existence of complex forms of certain nutrients such as pantothenic acid. In the present study the vitamin B_{12} potency of fresh and cooked meats has been determined by the rat growth method, by liver storage of the vitamin, and by microbiological methods. Preliminary results of certain phases of this study have been reported (Schweigert et al., '51).

EXPERIMENTAL

Animal assay

In the initial experiments several test diets were evaluated for rats and a purified diet (20% casein and 10% gelatin type) for chicks to determine the best basal ration for assay work. The results for straight-run, day-old chicks (Sky Line) with this basal ration (Wilkening and Schweigert, '47) plus 0.06% iodinated casein showed that the small growth differential be-

[1] Journal paper 52, American Meat Institute Foundation.

tween the groups fed the basal diet and basal diet + 16 μg vitamin B_{12} per kilogram (65 gm/week as compared to 78 gm/week) would not be satisfactory for our purposes. These tests were conducted for a three-week period after feeding the chicks the unsupplemented basal diet for a two-week period. Since

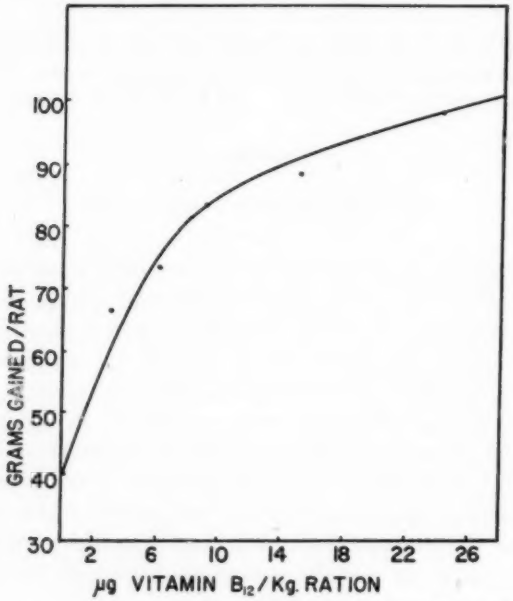

Fig. 1 Growth response of rats fed graded levels of crystalline vitamin B_{12} for a three-week period.

vitamin B_{12}-deficient chicks were not available, the rat assay for vitamin B_{12} was then investigated.

Preliminary tests with a purified casein-sucrose diet plus 0.06% iodinated casein [2] showed that a high mortality occurred among rats (Holtzmann) fed this diet either with or without vitamin B_{12} supplementation (Scheid, McBride and Schweigert, '50) and that no significant difference in growth rate was observed for the survivors regardless of the level of vitamin

[2] Protamone.

B_{12} fed. A corn-soybean oil meal basal diet plus 0.06% iodinated casein [3] was found to be satisfactory as a basal diet in that a graded growth response was obtained with increased levels of vitamin B_{12} and the mortality was low. The detailed composition of this ration has been described earlier (Scheid et al., '50) and is similar to that of the basal ration described by Register, Ruegamer and Elvehjem ('49).

Weanling, male, albino rats of the Holtzmann strain were kept in individual cages and fed and watered ad libitum. The basal diet was fed for a standardization period of two weeks. After the depletion period, the animals were arranged in subgroups as uniformly as possible based on weight and gain in weight for the previous two-week period. Each sub-group was fed the basal ration plus known amounts of crystalline vitamin B_{12} or materials that contained vitamin B_{12}. For the control animals crystalline vitamin B_{12} was added to the basal diet in the following amounts: 0, 2, 6, 9, 15, or 24 μg per kilogram ration. Seven rats were used in each group. The experiments were conducted for a period of three or 4 weeks and the results were calculated at the end of two, three or 4 weeks on experiment. The growth response with graded levels of vitamin B_{12} for a representative experiment is shown in figure 1.

Liver storage method

After completion of a rat growth experiment, all animals from selected groups were sacrificed by decapitation and the livers removed. The livers were assayed microbiologically, using *Lactobacillus leichmannii* 327 as the test organism according to the method previously described (Scheid and Schweigert, '51; Scheid, Andrews and Schweigert, 51). The vitamin B_{12} potency of the samples was then calculated from the results for the vitamin B_{12} potency of the livers from rats fed graded levels of vitamin B_{12}.

[3] See footnote 2, page 602.

Microbiological assay

The vitamin B_{12} potency of the test samples was determined after enzymatic treatment as described earlier (Scheid and Schweigert, '51). The more specific test organism *Euglena gracilis* was employed for the assay of vitamin B_{12} in beef liver. The details of this procedure have been reported (Scheid et al., '51).

Description and preparation of test samples

Two- to 10-pound samples of beef liver, kidney, round or rib cuts, fresh ham, and lamb leg were purchased directly from a packing plant, extraneous fat and connective tissue removed and the remaining material thoroughly ground to assure homogeneity. Portions of the ground sample were then taken for proximate and microbiological analysis and the remainder stored at $-5°$C.; in certain cases, portions were lyophilized and ether-extracted. The samples were added to the test diets at an appropriate level and the diets were then stored at $+2°$C. when not in use. For one of the experiments paired samples of beef rib (adjacent steaks $1\frac{1}{2}$ in. thick), fresh ham and lamb leg (right and left sides) were obtained. One of the paired samples was cooked in order to obtain data on the stability of the vitamin B_{12} during cooking. The beef rib steaks were broiled at 400°F. to an internal temperature of 150°F. The lamb leg and ham were roasted at an oven temperature of 325°F. to an internal temperature of 180°F. and 185°F., respectively. Liver extract (15 U.S.P. units per milliliter) was also included in order to study the vitamin B_{12} potency of a preparation high in vitamin B_{12} activity.

RESULTS AND DISCUSSION

A good minimum and maximum response to vitamin B_{12} were obtained in these experiments; the average gain per week during the three-week period for the basal group was 13 gm and that for the groups fed the basal diet plus 24 μg vitamin B_{12} per kilogram of ration was 32.6 gm (fig. 1). The average

weekly gain for all other groups was well within the range of the standard controls except for a group fed the basal ration plus 18 gm fresh liver per kilogram ration. The average weekly gain for this group was 34.3 gm. The results for the rat assay determined at different levels of the test sample and for varying times on experiments are presented in table 1. These studies were conducted in order to evaluate more critically the variation in results within an experiment. The variations

TABLE 1

Effect of level of test sample and time on experiment on the vitamin B_{12} potency of meats determined by the rat growth assay

MATERIAL ASSAYED		% PROTEIN	SUPPLEMENT PER KG	μG B_{12}/100 GM. CALC'D. TO FRESH BASIS		
				0–2 wk.	0–3 wk.	0–4 wk.
			gm			
Beef round	Fresh	21.42	150	1.8	3.7	
	Dry	88.05	30	3.1	4.9	
	Dry	88.05	50	2.3	4.1	
Pork ham	Dry	88.46	40	1.9	1.8	
	Dry	88.46	60	1.3	1.4	
	Fresh	14.3	120	1.8	1.3	1.2
	Fresh	14.3	200	1.5	1.2	1.3
Lamb leg	Fresh	16.73	50	8.0	6.5	7.6
	Fresh	16.73	100	7.8	8.6	5.6
Kidney	Fresh	14.76	10	45.0	44.5	40.0
	Fresh	14.76	15	37.3	33.2	30.7

noted indicate the desirability of testing the samples at more than one level, and that this procedure should be followed in animal assays of this type.

The data obtained for three rat assays are summarized in table 2. The supplements were also assayed microbiologically with *L. leichmannii* 327, and for one experiment by the rat liver storage method (table 2). These data show that beef liver contains 20 to 50 times and kidney 10 times as much vitamin B_{12} as muscle cuts. Beef and lamb muscle cuts also contain

somewhat greater quantities of vitamin B_{12} than the pork muscle cuts used in this study.

Studies on the different methods for estimating vitamin B_{12} indicated the following: (1) the rat growth and liver storage

TABLE 2

Comparison of rat growth and liver storage using microbiological assay for vitamin B_{12} [1]

SAMPLE	SAMPLE NO.	% PROTEIN	ANIMAL ASSAY		MICRO-BIOLOGICAL
			Growth	Liver storage	
			μg B_{12}/100 gm-fresh basis		
Experiment 1					
Beef liver	33		30		31
Beef round	34		4.5		3.4
Experiment 2					
Pork ham	36	17.90	1.6	2.5	0.9
Beef round	35	21.41	4.2	7.3	1.3
Beef liver	48	21.58	120	200	40.9
Beef kidney	47	15.66	55	54	18.5
Lamb leg	46	17.59	6.6	3.4	1.7
Experiment 3					
Pork ham	131	14.30	1.4		1.9
Cooked pork ham	132	18.46	2.3		2.1
Beef rib	133	16.85	2.5		2.9
Beef rib (cooked)	134	19.65	4.4		2.7
Beef liver	138	21.17	> 500		99
Beef kidney	137	14.76	40		14.6
Lamb leg	135	16.73	7.4		2.7
Lamb leg (cooked)	136	20.63	7.5		2.3
Injectable liver extract			13.0 [2]		15.5 [2]

[1] The rat assay in experiments 1 and 2 was conducted for three weeks and in experiment 3 for 4 weeks.

[2] Micrograms per milliliter.

method of assay gave higher values for vitamin B_{12} than did the microbiological method in the second experiment; (2) the values for fresh and cooked beef, pork muscle cuts and liver extract were in good agreement for the animal and microbiological assays in the third experiment, and for both beef liver and round in the first experiment, but the values by the rat

growth assay were again higher for the other samples; (3) the value for beef liver in the third experiment was unusually high as compared to that obtained by microbiological assay. This sample was found to contain 77 μg of vitamin B_{12} per 100 gm by the *Euglena gracilis* assay (Scheid et al., '51). Good agreement has been observed for the rat growth as compared to the microbiological assay for vitamin B_{12} in natural materials, however (Tappan et al., '50).

The general agreement in the values obtained by rat growth and liver storage measurements is of considerable interest. It should be pointed out that the calculations for the liver storage method were based on microbiological determination of the vitamin B_{12} potency of the livers of the rats fed crystal-

TABLE 3

Percentage retention of vitamin B_{12} after cooking

MATERIAL	PER CENT RETENTION OF VITAMIN B_{12}	
	Animal assay	Microbiological assay
Pork ham	127	84
Beef ribeye	155	82
Lamb leg	82	69

line vitamin B_{12} as compared to that of animals fed crude supplements. It may be concluded, therefore, that the vitamin B_{12}-active materials were as effectively utilized for liver storage as for growth when fed as these crude supplements.

Although only three paired cuts were tested for the stability of vitamin B_{12} during cooking, the retention of vitamin B_{12} during cooking (table 3) was 70% or more, based on either method of assay. The higher retentions noted for the animal assay as compared to the microbiological method indicate that either vitamin B_{12} from the uncooked meat was not completely available to the animal or that the liberation of vitamin B_{12} from cooked meat for the microbiological tests was less effective than that for the raw meat.

On an over-all basis, the higher results for vitamin B_{12} in beef liver observed in the third experiment with the rat assay,

as compared to the microbial assay, suggested that beef liver contained additional growth factors that were measured as vitamin B_{12} by the rat assay. Two other observations were also in accord with this possibility: the results with *Euglena gracilis* and *L. leichmannii* were in good agreement and the growth rate observed for rats fed beef liver exceeded the rate observed for those fed the highest vitamin B_{12} supplements to a slight extent. The higher rate of growth was evident for the first through the 4th week on experiment. A subsequent experiment was performed with rats that were fed the basal ration plus 50 μg vitamin B_{12} per kilogram of ration. Additional supplements of beef liver (5 to 100 gm per kilogram of ration), kidney (100 gm), or muscle cuts (100 gm) were tested with 10 rats in each group. No significant increase in the rates of gain was obtained with the addition of any of these supplements over that observed for vitamin B_{12}. Therefore, with the level of iodinated casein used (0.06%), no requirement for additional factors was observed with optimum vitamin B_{12} supplements. These levels of thyroid-stimulating material and of liver (dry basis) are considerably lower than those used by others in demonstrating the requirement for additional factors in liver (Betheil and Lardy, '49; Ershoff, '49). It is likely that factors in liver (and other meats) may have stimulated the rate of growth in the presence of subminimum levels of vitamin B_{12}, as would be the case for the tests conducted with these supplements using a rat assay.

An alternate explanation for these findings should not be overlooked. The lower results obtained in most cases microbiologically may be due to incomplete release of the vitamin, although extensive tests have been conducted to determine conditions for maximum release of the vitamin from bound forms. The limitations of both the rat assay as used here, with iodinated casein in the basal ration, and also of the microbiological assay, are clearly recognized. The use of the animal assay as a reference standard for comparison with shorter chemical or microbiological methods may not always be valid.

SUMMARY

Studies on the rat growth, liver storage and microbiological methods of analysis for vitamin B_{12} in meats have been conducted. The results obtained for the rat assay and microbiological assay were in good agreement for beef and pork muscle cuts, but the results by the rat growth and liver storage assay were considerably higher for lamb leg, beef liver and beef kidney. Beef liver was found to contain 20 to 50 times and beef kidney 10 times as much vitamin B_{12} as muscle cuts. Preliminary studies indicate that over 70% of the vitamin B_{12} is retained during cooking of pork, beef and lamb cuts. Limitations of the animal and microbiological methods for analysis for vitamin B_{12} are discussed.

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DEPOSITION OF FLUORINE IN THE BONES AND TEETH OF THE GROWING RAT

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FOUR FIGURES

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Only a limited number of reports have appeared on the effect of age on fluorine retention in bones and teeth, either when the fluorine exposure is continuous throughout life, or when it is instituted at various ages during the life span.

Lawrenz, Mitchell and Ruth ('40) found that "growing rats adapt themselves to the continuous ingestion of low levels of fluorine by excreting greater and greater proportions of the ingested fluorine in feces and urine," indicating that less fluorine was retained by aging skeletal and dental tissues. The fluorine excretion appeared also to approach a maximum or plateau value, which suggests a stabilized concentration of fluorine in the calcified tissues and a reduction in their capacity for fluorine retention.

A diminishing retention of skeletal fluorine with increasing age is also apparent from the data of Ellis and Maynard ('36). Over a 168-day period during which 8 p.p.m. fluorine were added to the diet, approximately 70% of the fluorine in rats' "leg bones" accumulated during the first third (56 days) of the fluorine exposure period. In another comparison in which 12 p.p.m. fluorine were added to the rats' diet, 55% of the total fluorine accumulated during the first third (56 days) of the exposure period.

Glock et al. ('41) fed approximately 200 p.p.m. fluorine in the diet to 6-week-old rats for 90 weeks, sacrificed representa-

tive rats at irregular intervals and analyzed the "long bones" for fluorine. The data fitted a logarithmic curve of diminishing increment, suggesting that a constant level of fluorine retention is attained with high as well as low levels of intake of fluorine.

Jackson, Tisdall, Drake and Wightman ('50) administered a diet containing approximately 5 p.p.m. fluorine to rats, sacrificed a representative number at 6-, 12-, and 18-month intervals and found that the total fluorine per rat carcass was essentially the same at each sacrifice period. Apparently fluorine was built up to a constant level and maintained so long as the dietary level was unchanged.

Data on the retention of fluorine in the calcified tissues in rats exposed to a given quantity of fluorine at various ages with no prior exposure to fluorine, appear to be limited to the work of (a) Jackson et al. ('50), who found that 6-month-old rats retained considerably less fluorine in the carcass than young rats started at 6 to 8 weeks of age, and (b) Savchuck and Armstrong ('51), who found that rats put on a fluorine regimen at 92 days of age retained less fluorine in their humeri than rats started at 27 days of age.

It was the purpose of this investigation to make a more comprehensive study of the effect of age on fluorine retention by means of periodic fluorine analyses of femurs, mandibles and teeth of rats undergoing continuous fluorine ingestion from 30 days to 450 days of age, and also by similar analyses to determine the response of the bones and teeth to fluorine introduced at various ages in the life of rats without prior exposure to fluorine.

PLAN OF THE EXPERIMENT

The investigation was composed of two studies, each of which was divided into 5 periods. At the start, Sprague-Dawley rats were housed 9 to a cage and received diet 550,[1] of the following composition: corn starch 66.5; whole milk

[1] Contains 1 p.p.m. F.

powder 27.0; yeast 5.0; cod liver oil 1.0; and salt mixture 0.5%. The salt mixture contained 500 gm of NaCl, 53.2 gm of ferric citrate trihydrate and 3.9 gm of copper sulfate pentahydrate.

In study I, the control rats received distilled water ad libitum, and the experimental rats received 10 p.p.m. F as NaF ad libitum in the drinking water, starting at 30 days of age.

Control and experimental rats were sacrificed after 60, 120, 180, 300 and 420 days on experiment. The femurs and mandibles of 6 control rats and 6 experimental rats, selected at random, were removed for analysis. The molar teeth of approximately 20 control rats were pooled, whereas the molar teeth of each experimental rat were sufficient for analysis. Fluorine analyses were made according to standard procedures (Willard and Winter, '33; McClure, '39) on the alcohol- and ether-extracted bones and teeth, dried overnight at 105°C.

In study II, control rats were fed the stock diet individually throughout the experiment. Starting at 30, 90, 150, 210 and 330 days of age, their drinking water contained 10 p.p.m. of fluorine as NaF. The fluid intake was measured and when 2,000 ml or 20 mg fluorine had been ingested, 6 rats most closely approximating the mean weight of the group were sacrificed.

The molar teeth, femurs and mandibles were removed and analyzed for fluorine as previously noted. Aliquot samples of the femurs and mandibles of the rats of studies I and II were ashed and analyzed for calcium (Association of Official Agricultural Chemists, '50a) and phosphorus (A. O. A. C., '50b).

RESULTS

Study I

To establish the initial fluorine content, a pooled sample of molar teeth, femurs and mandibles of 30-day old rats was analyzed for ash and fluorine. The relationship of the per

cent fluorine in the ash of the molar teeth, femurs, and mandibles to duration of fluorine exposure is presented graphically in figure 1 and similar data for total fluorine are presented graphically in figure 2.

In general, there was only a slight change in the per cent fluorine in the ash of control rats' bones and teeth during study I. The small increases in total fluorine in these tis-

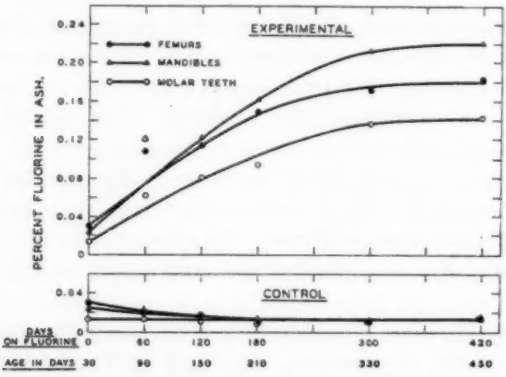

Fig. 1 Per cent fluorine in the ash of the bones and teeth of growing rats receiving 10 p.p.m. fluorine as NaF for 420 days starting at 30 days of age.

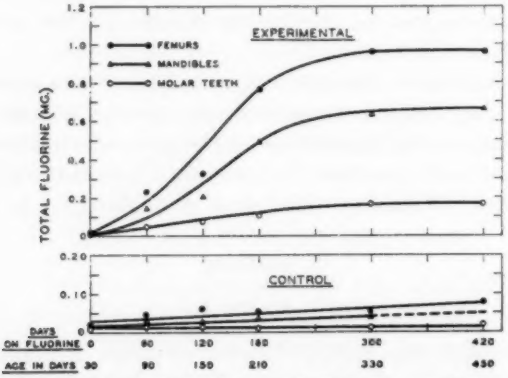

Fig. 2 Fluorine content of the bones and teeth of growing rats receiving 10 p.p.m. fluorine as NaF for 420 days starting at 30 days of age.

sues were due mainly to normal weight gains. As is shown in figure 2, those control rats which received only 1 p.p.m. fluorine from their basal diet increased their total fluorine two-fold, 4-fold, and three-fold in the molar teeth, femurs, and mandibles, respectively, by the end of the 450 days.

As is shown in figures 1 and 2, both the per cent fluorine and the total fluorine of the molar teeth, mandibles, and femurs of the experimental rats underwent marked increases up to 330 days of age; i.e., 300 days on a fluoride regimen of 10 p.p.m. fluorine in their drinking water. Further deposition of fluorine, however, was not apparent at the end of 450 days. Apparently a stabilized or saturation level of fluorine in the rats' calcified tissues was reached at about 330 days of age; i.e., near the end of the period of rapid growth.

By the end of 450 days of age, at which time the rat has already reached maturity, the total fluorine in the test rats had multiplied 20 times, 50 times, and 40 times in the molar teeth, femurs, and mandibles, respectively, as compared with their initial fluorine content. These would appear to represent maximum increases in fluorine under these conditions of intake at a level of 10 p.p.m. in the drinking water.

During the experimental period of 420 days there was no appreciable change in the calcium and phosphorus content of the femurs and mandibles of the rats receiving 10 p.p.m. fluorine. The calcium content of the femur and mandible ash averaged 39.29 $\pm$ 1.16% and 39.09 $\pm$ 1.11%, respectively, and the phosphorus content averaged 18.82 $\pm$ 0.79% and 18.88 $\pm$ 1.13%, respectively.[2]

Study II

The purpose of study II was to observe the deposition of fluorine in the molar teeth, femurs and mandibles of rats receiving equal amounts of fluorine as sodium fluoride at different ages, with no previous fluorine exposure. These data are presented in figures 3 and 4.

[2] Values are expressed as mean $\pm$ standard deviation.

A pronounced decrease was apparent in the per cent and total fluorine deposited in bones and teeth with increasing age; i.e., old rats without prior exposure to fluorine stored considerably less fluorine in their bones and teeth than younger

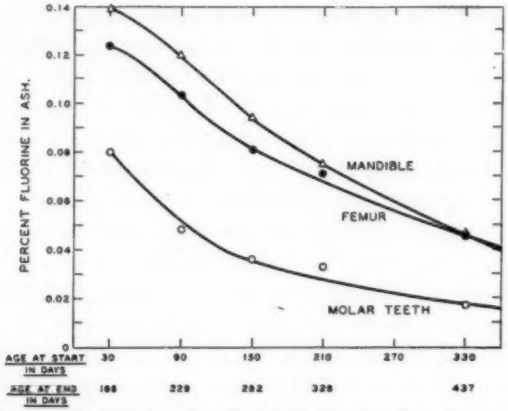

Fig. 3 Per cent fluorine in the ash of the bones and teeth of growing rats receiving 20 mg of fluorine as NaF at different ages, with no prior exposure to fluoride.

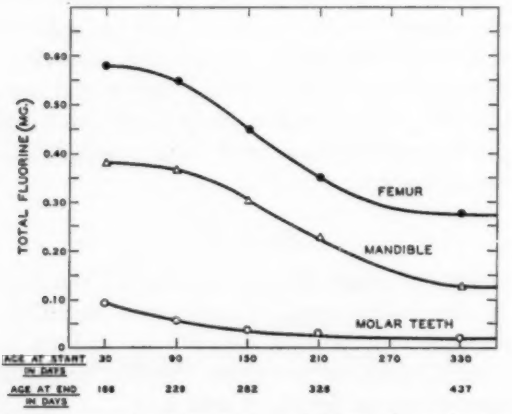

Fig. 4 Fluorine content of the bones and teeth of growing rats receiving 20 mg of fluorine as NaF at different ages, with no prior exposure to fluoride.

animals. The ratios of the per cent and total fluorine found in the bones and teeth of the rats of group V (oldest rats) to those of the rats of group I (youngest rats) were, respectively, as follows: for molar teeth, 0.22 and 0.20; for femurs, 0.37 and 0.48; and for mandibles, 0.32 and 0.34.

It is of interest to note also that the femurs and mandibles contained a similar per cent of calcium and phosphorus, and this remained unchanged despite a variable fluorine content. Thus the femurs and mandibles averaged 38.85 $\pm$ 1.74% calcium and 39.03 $\pm$ 1.42% calcium, respectively, and the phosphorus content averaged 18.43 $\pm$ 0.81% and 18.56 $\pm$ 0.70%, respectively.[3]

DISCUSSION

The data obtained in both study I and study II agree with previous evidence and emphasize the fact that the animal body, as typified by the rat, does not store fluorine with fixed regularity and constancy throughout life. Retention of fluorine by the bones and teeth in these rats appeared to be primarily related to growth; i.e., the fluorine deposition curves shown in figures 1 and 2 follow the general pattern of a normal growth curve. During the period of rapid growth, both the per cent and total fluorine of the molar teeth, femurs and mandibles increase at a relatively rapid rate. In the mature rat, however, fluorine storage does not increase with increase in age, as shown by essentially similar concentrations of fluorine in the bones and teeth of rats at 330 and 450 days of age. It appears that the data of figures 1 and 2 describe a relationship of diminishing increment between fluorine deposition and time. This is in accord with the data of Lawrenz et al. ('40), previously referred to.

Interpolation from the curves of figure 2 shows that 50% of the fluorine retained by the molar teeth over a 420-day experimental period was accumulated during the first third (120 days) of the fluorine exposure period. During this same interval, the mandibles and femurs accumulated 57% and

[3] See footnote 2, page 615.

66% of the total fluorine retained. The latter value for femurs is in good accord with the data of Ellis and Maynard ('36), who found that approximately 70% of the fluorine in the rats' "leg bones" accumulated during the first third of the fluorine exposure period when 8 p.p.m. fluorine were added to the diet.

Although the rats in study II had no prior fluorine exposure, as contrasted with the continuous fluorine exposure of the animals of study I, these rats (study II) showed a similar loss of ability to retain fluorine with advancing age.

By reference to figures 2 and 4 it is possible to estimate the effect of prior exposure to fluorine on its subsequent deposition. As shown in figure 2, only a very slight amount of fluorine is deposited in the bones and teeth of rats during the interval between 330 and 437 days of age when the animals receive 10 p.p.m. F continually throughout life. During this same interval of time, rats with no prior exposure to fluorine accumulated appreciable amounts in the femurs and mandibles, as is shown in figure 4, when compared to the fluorine deposition in the control rats, as shown in figure 2.

Thus the 330-day-old rats of study II (no prior exposure to fluorine) stored 0.22 mg and 0.09 mg in the femurs and mandibles, respectively; whereas similar rats of study I (continuous exposure to fluorine) stored only 0.02 and 0.01 mg of fluorine, respectively.

The molar teeth of rats 330 days of age with no prior exposure to fluorine and receiving 10 p.p.m. fluorine contained the same amount of fluorine at 437 days of age as those of control rats of the same age.

These observations indicate, therefore, that the bones of the mature rats in study I were exhausting their fluorine deposition potential, whereas rats of similar age (study II) with no prior exposure to fluorine retained their ability to store fluorine, albeit at a much reduced capacity compared to that of rapidly growing rats. It is interesting to note that a similar situation has been demonstrated with regard to skeletal

deposition of radioactive calcium in young and adult rats by Copp et al. ('51) and Hansard et al. ('51), who showed that skeletal uptake of calcium 45 is greater in growing rats than in older ones.

SUMMARY

Rats 30 days of age received 10 p.p.m. F as sodium fluoride for a period of 420 days. At various intervals during this period representative rats were sacrificed and the molar teeth, femurs and mandibles were analyzed for fluorine. It appears that the growing rat shows a diminishing ability to store fluorine in the bones and teeth with advancing age and after the period of rapid growth accumulates little or no additional fluorine.

Rats put on a fluoride regimen at various ages of from 30 to 330 days without prior exposure to fluorine, and receiving equal quantities of fluorine, also showed a pronounced decreasing capacity to incorporate fluorine in the bones and teeth with advancing age.

The results agree with previous evidence that the animal body accumulates fluorine at a comparatively rapid rate during growth, but reaches a relatively constant level of skeletal fluorine at maturity. This is maintained throughout the remaining years of life so long as the fluorine intake remains stabilized.

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REPRODUCTION STUDIES WITH DOGS

I. EFFECT OF A CORN-SOYBEAN MEAL-ALFALFA DIET [1]

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ONE FIGURE

(Received for publication March 17, 1952)

INTRODUCTION

Although the nutritional requirements for growth and maintenance of the dog are well known, very little information concerning the requirements for reproduction and lactation is available. Elvehjem et al. ('44) have reported the effect of vitamin E on reproduction in dogs fed mineralized milk diets, Koehn ('42) has given the composition of some "home formulated" diets which were tested for their ability to support reproduction and lactation, and Robinson ('47) has reported a series of feeding trials of commercial dog foods in which reproduction and lactation were used as the primary criteria. However, there is no published work on the basis of which one can make a critical appraisal of the nutritional factors necessary for successful reproduction and lactation in dogs.

In 1947 experiments were begun to determine the effect on reproduction and lactation in dogs of a purified diet, a

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the American Feed Manufacturers Association, Inc., Chicago, Illinois, and the Brewers' Yeast Council, Inc., St. Louis, Missouri.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the generous supply of crystalline vitamins and for the vitamin B_{12} concentrate, and to the Abbott Laboratories, North Chicago, Illinois, for the haliver oil.

corn-soybean meal-alfalfa diet, and a series of experimental diets composed of commercially available ingredients. Only the experiments in which the corn-soybean meal-alfalfa diet was used as the basal diet will be presented at this time.

Corn-soybean meal-alfalfa diets have been used extensively in studying the nutritional requirements for reproduction and lactation in swine and rats (Ross, Phillips and Bohstedt, '42a, b; Cunha, Ross, Phillips and Bohstedt, '44; Ross, Phillips, Bohstedt and Cunha, '44; Spitzer and Phillips, '46; Maruyama and Phillips, '48; Nell and Phillips, '50). Until recently, normal reproduction and lactation could be obtained only upon the addition of certain natural supplements such as liver, crude casein and condensed fish solubles. Nell and Phillips ('50) found that the addition of vitamin B_{12} would allow for normal reproduction of rats fed the corn-soybean meal-alfalfa diet. The necessity of vitamin B_{12} for reproduction has also been demonstrated by the work of Schultze ('49) and Sure ('51).

The present studies were undertaken with dogs in an effort to learn more about the nutritional requirements for reproduction. However, in addition, the practical value of an all-plant ration suitable for growth, maintenance, reproduction, and lactation in dogs is self-evident.

EXPERIMENTAL

Twenty-one weanling bitches (cocker spaniels, beagle hounds, and wire-haired terriers) and 6 proven studs were obtained from reputable commercial kennels throughout the Middle West.

Before being admitted to the laboratory each animal was inoculated against distemper, dewormed, and washed in a DDT emulsion bath.

On admission to the kennels the animals were housed in individual cages with expanded metal floors. Each bitch was placed on an assigned experimental diet, which she continued to receive throughout the entire experimental period unless

otherwise indicated. Insofar as possible, litter-mate grouping was practiced in forming the experimental groups.

All dogs were given food and water ad libitum, and each was given 10 drops of halibut liver oil weekly. The basal diet (CSA) consisted of ground yellow corn 58.0, soybean oil meal 32.0, alfalfa meal 5.0, a salt mixture 2.0, and cottonseed oil 3.0%. One gram of irradiated yeast and 2.0 gm niacin were added per kilogram of diet. The salt mixture was composed of $CaCO_3$ 50.00, NaCl 24.00, $CaHPO_4$ 24.00, $MgSO_4$ 1.00, $MnSO_4$ 0.95 and KI 0.05%.

In this study 6 experimental groups were used. The basal diet was supplemented at the expense of the whole ration in the following manner: group I, no supplement; group II, 6% dried brewers' yeast; group III, vitamin B_{12} concentrates (calculated to furnish 60 μg vitamin B_{12}/kg diet); group IV, 20 μg crystalline vitamin B_{12}/kg diet; group V, 5% condensed fish solubles; group VI, 10% fresh beef liver. In addition, one dog in group III was transferred to a ration containing 60 μg crystalline vitamin B_{12}/kg diet. The stud dogs were fed a commercial dog food, which was supplemented with 10% fresh beef liver.

When the dogs became fully grown, they were observed for estrus. As each bitch came into heat she was bred at least twice in order to assure fertilization. All bitches were bred every estrus throughout the entire experiment.

Food and water containers were normally attached to the side wall of the cage to minimize loss of food and to prevent the pups from eating from them except during the last two weeks of lactation, when the containers were placed on the floor.

Individual records were kept of daily food consumption, weekly weight of bitch, estrus, general condition, and weekly weight of pups.

RESULTS

The growth rates of comparable pups in all experimental groups were essentially equal. The growth data presented

in figure 1 illustrate these results. After reaching maturity they were maintained on the same diets for over two years and remained in a normal and healthy condition. From these observations it is concluded that the basal diet is adequate

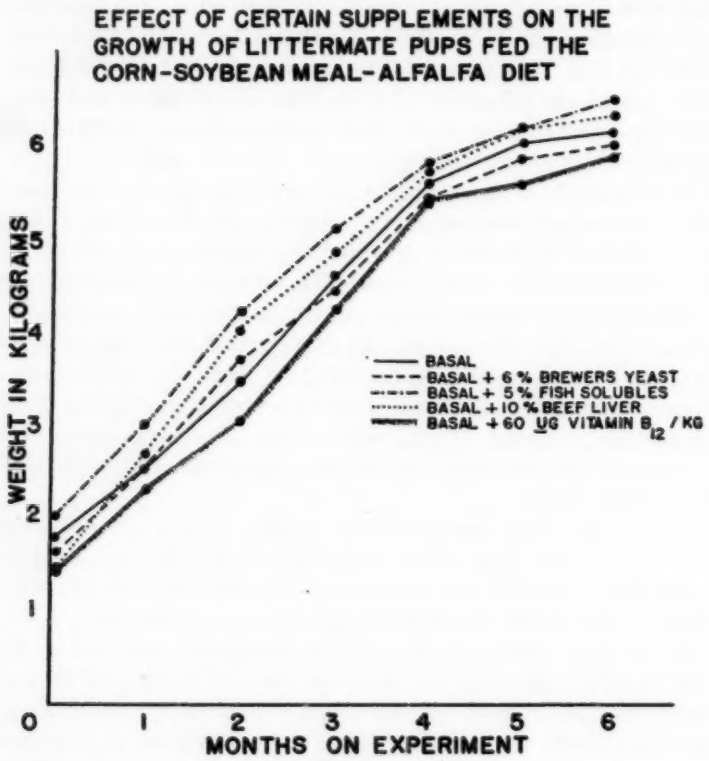

Figure 1

for growth and maintenance in dogs, since no differences were noted between dogs receiving the unsupplemented and supplemented diets.

Contrary to the results obtained with respect to growth and maintenance, marked differences were obtained in the different experimental groups when reproduction and lacta-

tion were used as the criteria. The results of these reproduction studies are summarized in table 1.

Only bitches which received supplements of 10% fresh beef liver, 5% condensed fish solubles, 20 μg crystalline vitamin B_{12}/kg diet, or 60 μg crystalline vitamin B_{12}/kg diet were able to reproduce and raise their young successfully.

The dogs which received the basal diet alone appeared to have a normal estrus and would breed, but generally they would not produce young. Only one of the bitches fed this diet produced a litter. These pups were very small and poorly developed. They appeared to have been born before normal whelping age, although the gestation period of the bitch was 64 days.

Pups of normal size and apparent vigor were produced by the animals which received supplements of yeast or vitamin B_{12} concentrate. They appeared healthy during the first 12 to 24 hours after birth, then stopped nursing and became progressively more lethargic until death. Just prior to death tetany was often observed, which seemed to indicate a magnesium deficiency (Follis, '48). However, the blood magnesium levels of these pups were found to be the same as those in normal pups of the same age.

Most of the pups from bitches in groups II and III were observed to be in a general hyperemic condition at death, a condition similar to the uremia syndrome reported by Schultze ('49). Blood urea levels were determined on some of these animals and were found to be within the range for normal pups. Several pups were autopsied immediately after death and specimens of major organs and endocrine glands were taken for histological examination. Comparison with tissues of normal pups of similar age did not reveal any important differences.

Attempts to determine the quality of the milk from the bitches were made by giving them normal, two-day-old pups to nurse. Most of these trials were unsuccessful because the bitches would not accept foster pups. However, one pup was successfully weaned in this manner. This seemed to

TABLE 1

The effect of certain supplements on reproduction and lactation in bitches fed the corn-soybean meal-alfalfa diet

SUPPLEMENT	NUMBER BITCHES	NUMBER BREEDINGS	NUMBER LITTERS BORN	NUMBER LITTERS WEANED	NUMBER PUPS BORN	AVERAGE WEIGHT AT BIRTH	NUMBER PUPS WEANED	AVERAGE WEIGHT AT WEANING
						gm		*gm*
None	6	8	1	0	6	122	0	....
6% brewers' yeast	3	4	2	0	8	267	0	....
60 μg vitamin B_{12}/kg diet (concentrate)	3	6	6	0	25	252	0	....
20 μg vitamin B_{12}/kg diet	2	4	4	4	18	266	12	1,262
5% condensed fish solubles	3	4	3	3	8	276	7	1,527
10% beef liver	4	5	4	4	20	263	17	1,321
60 μg vitamin B_{12}/kg diet	1[1]	1	1	1	5	280	4	1,425

[1] From group III.

indicate that the milk was not the prime factor in the death of the pups.

DISCUSSION

From the results obtained in this study it appears that the nutritional requirements for growth and maintenance of the dog are furnished by the corn-soybean meal-alfalfa diet described; however, this diet does not contain all of the nutrients necessary for reproduction. Successful reproduction and lactation were obtained when the diet was supplemented with 10% fresh liver, 5% condensed fish solubles, 20 μg crystalline vitamin B_{12}/kg diet, or 60 μg crystalline vitamin B_{12}/kg diet. These results are in substantial agreement with those previously obtained with rats and swine. Since all of the supplements which adequately fortified the basal diet contained vitamin B_{12}, it appears that the dog requires this vitamin for reproduction and lactation.

Bitches fed the basal diet alone and the basal diet supplemented with 6% brewers' yeast or vitamin B_{12} concentrate, calculated to furnish 60 μg vitamin B_{12}/kg diet, never completed a normal reproductive cycle. However, both yeast and the concentrate had a beneficial effect on the number of pups born and on their birth weight. Since brewers' yeast contains no vitamin B_{12}, it must be concluded that this favorable effect on embryonic development was mediated through some mechanism which did not involve a dietary source of vitamin B_{12}.

It is very difficult to interpret the results obtained with the vitamin B_{12} concentrate. One bitch which had two unsuccessful litters when fed the concentrate produced a normal litter when changed to a diet containing 60 μg crystalline vitamin B_{12}/kg diet. The concentrate contained a crude vitamin B_{12} preparation, charcoal and soybean flour. According to potency claims, it was supposed to furnish 12.5 mg vitamin B_{12}/lb. and all calculations were made on this basis; however, an assay of this material showed it to contain only 2 mg vitamin B_{12}/lb. Thus, it is likely that the dogs in group

III did not receive 60 μg vitamin B_{12}/kg diet. As a matter of fact, some of these dogs may have received as little as 10 μg vitamin B_{12}/kg diet. If results obtained with rats can be extended to dogs, this amount of vitamin B_{12} should have been adequate. One can only speculate about the conflicting results obtained. It is possible that the vitamin B_{12} in the concentrate was not available to the dogs, that the concentrate contained unknown injurious substances, that 10 μg vitamin B_{12}/kg diet were not sufficient for successful reproduction, or that a combination of these factors was responsible for the results obtained. These observations suggest the need for a critical appraisal of work reported in the literature involving the use of crude vitamin B_{12} preparations.

Considerations of the cost and composition of the corn-soybean meal-alfalfa diet make it appear very attractive as a practical dog food. However, the diet is not acceptable to most dogs unless they are raised on it from weaning. This problem can usually be overcome by substituting, over a period of two weeks, larger and larger increments of this ration for the one previously used.

SUMMARY

These studies indicated that a diet composed of ground yellow corn, soybean oil meal, alfalfa meal, cottonseed oil, a mineral mix, niacin and irradiated yeast was adequate for the growth and maintenance of dogs.

This diet when supplemented with 20 or 60 μg crystalline vitamin B_{12}/kg, 5% condensed fish solubles, or 10% fresh beef liver was adequate for reproduction and lactation in dogs.

The basal diet alone and the basal diet supplemented with 6% brewers' yeast or a vitamin B_{12} concentrate would not support bitches through a reproductive cycle. The apparent discrepancy between the results obtained with crystalline vitamin B_{12} and the crude concentrate used is discussed.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to E. S. Robajdek for his help in caring for some of the dogs, and to L. Michaud, Merck and Co., for the assay of the vitamin B_{12} concentrate.

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THE REQUIREMENT FOR ISOLEUCINE AND THE ACTIVITIES OF ITS ISOMERS FOR THE GROWTH OF TURKEY POULTS

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ONE FIGURE

(Received for publication April 14, 1952)

Isoleucine has been shown to be required for optimum growth in chicks (Grau and Almquist, '44; Almquist and Grau, '44; Hegsted, '44; Grau and Peterson, '46). There have been no reports on the isoleucine requirement of turkey poults. Several experiments were undertaken to determine the amount of isoleucine needed by poults and the activity of the various isomers of isoleucine.

EXPERIMENTAL

Bronze poults were fed a practical starting ration for approximately one week before being divided into experimental groups of comparable weight. There were from 5 to 7 poults per group. They were raised in electrically heated batteries and were supplied feed and water ad libitum. The poults were weighed at frequent intervals and were continued on experiment for from 8 to 14 days in the various trials.

The basal ration contained the following: blood meal 28.0 gm; gelatin 5.0 gm; calcium gluconate 5.0 gm; cellulose [1] 3.0 gm; salt mixture (Kratzer et al., '49) 2.5 gm; tricalcium phosphate 2.5 gm; soybean oil 2.5 gm; vitamin mixture [2]

[1] Cellu flour.

[2] Pteroylglutamic acid was kindly supplied by Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

(Kratzer et al., '49) 2.0 gm; dicalcium phosphate 1.5 gm; fish oil (2,250 A, 300 D) 1.0 gm; choline chloride 0.3 gm; L-cystine 0.2 gm; DL-methionine 0.2 gm; inositol 0.1 gm; vitamin B_{12} concentrate [3] 0.1 gm; alpha-tocopherol acetate 2 mg; and corn starch to make up to 100 gm. Various levels of isoleucine were added to the basal ration at the expense of cornstarch. The isoleucine samples used were commercial preparations from three different manufacturers.[4] All samples except sample 3 were found to have no optical rotatory activity.[5]

The blood meal contained 0.79% L-isoleucine as determined by the method of Kuiken et al. ('43) using *Lactobacillus arabinosus,* while the gelatin contained 1.29%. From these values it may be calculated that the basal ration contained 0.29% L-*isoleucine.* The ration contained 26.8% crude protein. Growth at optimum levels of isoleucine was slightly less than that obtained with control groups fed a practical starting ration.

RESULTS AND DISCUSSION

DL-isoleucine (sample 1) was fed at various levels in the first two experiments. The poults actually lost weight when no supplement was added and gained until maximum growth was obtained with approximately 2.0% added DL-isoleucine (fig. 1). The gains are plotted as averages of male and female average daily percentage body weight gains.

After two experiments our supply of DL-isoleucine sample 1 was exhausted and sample 2 was then used. It is apparent from figure 1 that approximately 1.0% of this sample was needed for optimum growth.

Since there was a wide discrepancy in the response of the poults to isoleucine samples 1 and 2, other samples were ob-

[3] APF 3 containing 12.5 mg vitamin B_{12} per pound, kindly supplied by Merck and Company through the courtesy of Dr. D. F. Green.

[4] Sample 3 was kindly supplied by the Dow Chemical Company, Midland, Michigan, through the courtesy of Mr. Chester Otis.

[5] We are grateful to Dr. E. P. Painter, of the Chemistry Division, for the optical rotation measurements.

tained and tested for poult growth-promoting activity. Samples 3, 4 and 5 were fed at levels to give suboptimum growth to compare their activity directly with that of sample 2, which was arbitrarily assigned a relative growth-promoting value of 100 (table 1). At least two groups were used for testing the activity of each sample, except for sample 1, the value for which was obtained directly from figure 1.

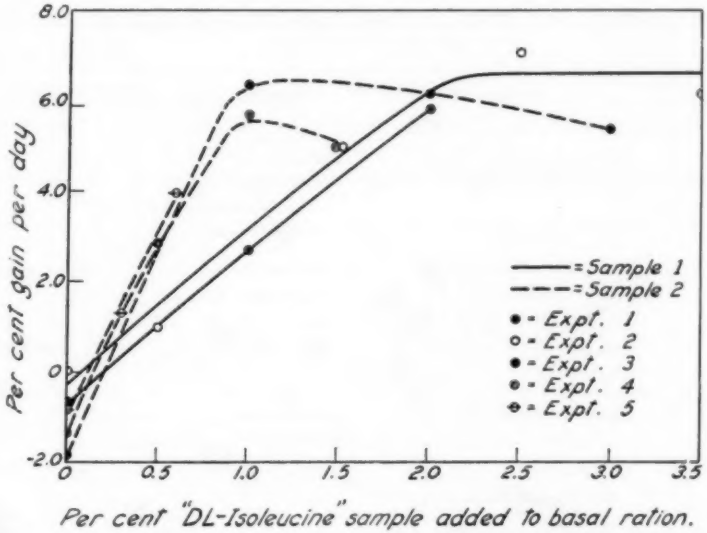

Fig. 1 The effect of different lots of DL-isoleucine upon the growth of poults.

Greenstein et al. ('51) have given the composition of samples of isoleucine identified by lot numbers which correspond to our samples 2 and 4. Sample 1 is probably similar to their category A, since it was purchased at approximately the same time.

Samples 2 and 4 gave approximately equal activity, which could be explained if the activity in each case were due only to the L-isoleucine. Similarly, the fact that sample 1 gave half as much activity as sample 2 might be due to L-isoleucine's being the only active form among the 4 isomers. Sample 3,

with a slightly higher L-isoleucine content, gave slightly better growth than any of the equal mixtures of L-isoleucine and either D-isoleucine or D-alloisoleucine. The results indicate that L-isoleucine is the only isomer which possesses growth-promoting activity for the poult. This agrees with the results of Greenstein et al. ('51), who showed that rats could use

TABLE 1

Relative activity of various isoleucine preparations for promoting the growth of turkey poults

SAMPLE NUMBER	DESCRIPTION	PROBABLE FORMS	RELATIVE GROWTH-PROMOTING ACTIVITY
			%
1	DL-isoleucine, Nutritional Biochemicals Corp. Lot 2888	L-isoleucine D-isoleucine L-alloisoleucine D-alloisoleucine	50
2	DL-isoleucine, Nutritional Biochemicals Corp. Lot 8305	L-isoleucine [1] D-alloisoleucine	100
3	L-isoleucine (with D-alloisoleucine) [1] Dow Chemical Co. Lot 53322	56% L-isoleucine [2] 44% D-alloisoleucine	115
4	DL-isoleucine, Merck and Co. Lot 40761	L-isoleucine [1] D-isoleucine	99
5	DL-isoleucine, Nutritional Biochemical Corp. Lot 9717	Unknown	99

[1] According to Greenstein et al. ('51).

[2] According to the manufacturer, and calculation from optical rotation measurement.

only the L-isomer. Grau and Peterson ('46) observed that L-isoleucine was active and D-isoleucine inactive in the chick.

We may conclude from figure 1 that the amounts of samples 1 and 2 which need to be added to the basal ration for optimum growth are 2.0% and 1.0%, respectively. Assuming that these samples contain 25% and 50% L-isoleucine, respectively, the amount of added L-isoleucine which is needed is 0.50%. If we add to this the amount contained in the basal ration or 0.29%, the total requirement for optimum growth is found to

be 0.79% or approximately 0.8%. This is considerably higher than the chick requirement of 0.5% as determined by Grau and Peterson ('46).

SUMMARY

Young turkey poults require approximately 0.8% L-isoleucine in their diet for optimum growth. Various isoleucine samples were active in proportion to their L-isoleucine content, which indicates that the other stereoisomers are inactive for this species.

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